AD_____

AWARD NUMBER DAMD17-94-J-4372

TITLE: Splicing Variants Of Estrogen Receptor in Breast Cancer

PRINCIPAL INVESTIGATOR: Richard J. Miksicek, Ph.D.

CONTRACTING ORGANIZATION: New York State University At Stonybrook
Stonybrook, New York 11794

REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Annual (30 Sep 97 - 29 Sep 98)

4. TITLE AND SUBTITLE
Splicing Variants of Estrogen Receptor in Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4372

6. AUTHOR(S)
Richard J. Miksicek, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Research Foundation of SUNY
Stoney Brook, New York 11794

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Progress reported for year 4 of this award includes: 1) an analysis of ER-α mRNA variants in two cell lines (TG-1 and TG-3c) from the MCF10AT series (a model for preneoplastic breast disease), 2) PCR analysis of ER-α mRNA in M13 SV1 and R_2N_1 cells (SV40 immortalized normal human breast epithelial lines developed as a model for breast stem cells), and 3) continued characterization of selected ER-α splicing variants, focusing on non-canonical estrogen response elements (EREs). The findings extend our work to include two new model systems for premalignant breast disease. In both models, a diverse array of splicing variants are seen at the RNA level, but the major variants differ compared with the MCF-7 system. We continue to investigate the significance of these findings by analyzing the behavior of transfected reporter plasmids in the TG-1, TG-3c, and M13 cell lines. Our analysis of the ability of ER-α splicing variants to modulate gene expression via non-classical (ERE-independent) pathways has also progressed. Data from transfection studies exploring the behavior of selected splicing variants on four reporter constructs that lack consensus EREs are summarized. A variety of behaviors are observed, including reporters that are activated primarily by ERΔE3, primarily by ERΔE5, or by both. These studies provide the first convincing evidence that ER-α variants are able to mediate positive regulation of gene expression on a subset of genes that contain non-canonical hormone response elements. They provide further evidence that ER-α splicing variants can contribute both to positive and to negative regulation of transcription, suggesting that their impact on the growth, differentiation, and neoplastic transformation of the breast deserves continued study.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
65

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__X__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__X__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Richard J. Kracek 6/11/99

PI - Signature Date

Table of Contents

INTRODUCTION

Title: Splicing Variants of the Estrogen Receptor in Breast Cancer
ID No.: DAMD-1794-J-4372
PI: Richard J. Miksicek
Period: 10/01/97 through 09/30/98

Summary of Administrative Matters

This USAMRDC Breast Cancer Research Award was made to Richard J. Miksicek, who currently holds an appointment as Assistant Professor of Physiology and Member of the Cancer Center at Michigan State University (MSU). This report summarizes the fourth year of this award, through 09/30/98. At the time this grant was awarded, Dr. Miksicek was on the faculty at the State University of New York (SUNY) at Stony Brook. During the first year of this award, the P.I. accepted a new position (effective 07/01/95) in the Department of Physiology at MSU. At that time, permission was requested from the US Army MRMC through the awardee institution (The Research Foundation of SUNY) to effectively change the site of performance of this project from SUNY @ Stony Brook to MSU by establishing a research subcontract between these institutions. This research subcontract stipulated that Dr. Sandra Haslam (Professor of Physiology and Director of the MSU Breast Cancer Program) be named as Principal Investigator for the MSU subgrant with Dr. Miksicek continuing to serve as P.I. on the primary award to SUNY @ Stony Brook. In addition, Dr. Miksicek has been acting as Principal Co-Investigator at MSU with primary responsibility for the conduct of research and grant administration. This subcontract was accepted by both institutions on 03/28/96 to become effective retroactively to 07/01/95. Predictably, progress on this project was substantially delayed between 07/01/95 and 03/28/96 pending negotiation of the research subcontract and release of research funds at MSU. During 1996, the investigator's laboratory was re-established at MSU and the following research staff were recruited to resume work on this project. For the current reporting period (10/01/97 through 9/30/98):

Key Personnel:

Name	Degree	Role	Effort	Period of Service
MIKSICEK, Richard	Ph.D.	PI (SUNY) & Co-PI (MSU)	25%	09/94-present
ANKRAPP, David	Ph.D.	Postdoctoral Research Fellow	100%	07/96-09/98
BOLLIG, Aliccia	B.S.	Graduate Research Asst.	100%	10/95-present
MORRISON, Mary	M.S.	Research Asst.	15%	02/96-03/98

Dr. Miksicek received partial summer salary from this award for the period 6/98-8/98. Dr. Ankrapp and Ms. Bollig drew full salary support from this award during the reporting period. Ms. Morrison was supported primarily from a non-overlapping award (DAMD1794-J-4411), but she provided significant technical support to this project (until her retirement in 03/98), in the form of laboratory management, cell culture, media preparation, antibody preparation, DNA sequencing, and technical assistance. In addition, this award provided supplemental salary during year 4 for a part-time minority work study student, John Hicks. During 09/98, the PI requested a one year no-cost extension through SUNY at Stony Brook, the recipient of the primary award, in order to make up for time lost in moving this project from Stony Brook to MSU. Work planned for completion during this extension period will be largely limited to the remainder of Task 2b, involving functional analysis of the transcriptional stimulatory activity of ER-α splicing variants on non-classically regulated genes that contain estrogen-responsive AP-1 motifs.

Scope of the Project

Human breast tumors and tumor-derived cell lines contain a mixed population of estrogen receptor-α (ER-α) messenger RNAs (mRNAs), representing both correctly and aberrantly processed transcripts (1-3). The aberrant ER-α mRNAs are predicted to give rise to structurally altered receptor molecules that are likely to interfere with the normal estrogen response pathway present in breast tumor cells. Early reports speculated that these variants may provide a molecular explanation for the loss of estrogen responsiveness or the acquisition of resistance to tamoxifen and other estrogen antagonists (4,5), thereby reducing the effectiveness of hormonal adjuvant therapy of breast cancer. We received this award to undertake a detailed analysis of splicing variants of ER-α mRNA in selected human breast tumor specimens and to characterize the functional activity of these variants in transfected cells. During the course of this project, we have also attempted to refine techniques and develop reagents (both molecular and immunological) to enable further investigation of the clinical significance of these ER-α splicing variants.

The approach being used in this project involves amplification of complementary DNAs (cDNAs) using the polymerase chain reaction (PCR), followed by sequence analysis of the resulting clones. In parallel, plasmid vectors have been constructed to enable expression and functional characterization of selected ER-α splicing variants in both transiently and stably transfected cells. The original statement of work for this project included the following specific tasks:

Task 1, Identification of ER mRNA variants in breast tissue (months 1-24):

a. Preparation of oligonucleotide primers for the analysis of ER mRNA splicing variants and optimization of PCR amplification conditions.
b. PCR amplification of ER cDNAs prepared from tamoxifen-resistant tumors.
c. PCR amplification of Progesterone Receptor cDNAs prepared from tamoxifen-resistant tumors.
d. PCR amplification of ER cDNAs prepared from normal human breast tissue.

Task 2, Functional analysis of ER mRNA splicing variants (months 18-48):

a. Construction of ER expression plasmids harboring variant ER cDNAs.
b. Analysis of the transcriptional stimulatory activity or inhibitory activity of ER splicing variants by transient transfection.
c. Production and characterization of cell lines that stably express the "constitutive" ERΔE5 variant.

Work during the current reporting period involved primarily Tasks 1.d. and 2.b. For several reasons, the decision was made to substitute the M13 SV1 and MCF10A derivative cell lines as proxies for normal human breast tissue in Task 1.d.

EXPERIMENTAL METHODS

Cell lines and cell culture.

MCF10A/TG-1 and TG-3c cells, obtained from the Karmanos Cancer Institute (Detroit, MI), were cultured in a 1:1 mixture of Hams-F12:DMEM media containing 5% horse serum. M13 SV1 and M13 R_2N_1 cells, obtained from C.C. Chang (Dept of Pediatrics, MSU), were cultured as described in MSU media containing 10% fetal bovine serum. HeLa cells were cultured as described in previous annual reports in DMEM containing 10% calf serum. In addition, all cell lines were supplemented with 5 mM HEPES (pH 7.4), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Experiments involving DNA transfection, RNA extraction, and PCR analysis utilized cells that were subcultured fewer than 25 passages from receipt.

PCR analysis of ER-α mRNA splicing variants in premalignant breast epithelial cell lines.

Standard conditions were employed for PCR amplification of cDNA sequences using AmpliTaq DNA polymerase (GIBCO/BRL) and a Stratagene thermocylcer. Synthetic primers used for the current experiments have been described in previous reports. Reactions typically involved 35-40 cycles of amplification, beginning with 30 seconds of denaturation at 94° C, followed by a 60 second annealing step (56-66° C) and 60 to 300 seconds of elongation at 74° C, depending on the length of the interval to be amplified. Annealing temperatures were carefully selected for each primer pair and were held as stringent as possible to optimize for the specificity and efficiency of amplification. Total RNA (for use in RT/PCR experiments) was prepared using Trisolve reagent (GIBCO/BRL) according to the manufacturer's instructions. Single-stranded cDNAs were synthesized using MLV reverse transcriptase from 10 μg aliquots of total RNA, priming variously with oligo$(dT)_{15}$ or a lower strand ER-α specific primer. In general, amplification reactions began with the cDNA equivalent of 1 μg of total RNA. No consistent differences in PCR yield or product profile were noticed comparing cDNAs made with oligo(dT) or with an ER-specific RT primer. Products of PCR reactions were routinely cloned using InVitrogen's pCR2-TOP kit according to manufacturer's instructions. When the goal was to perform a clonal analysis of the products of key PCR experiments, representative cDNA libraries were established by picking individual bacterial transformants into 96 well microtitre plates containing LB medium, incubating overnight at 37° C, and freezing at -70° C after the addition of glycerol to 10%. Individual transformants were picked, grown up, and used for miniprep DNA isolation. Plasmid DNA was subjected to a battery of restriction enzyme digests and, where appropriate, DNA sequence analysis.

Transient transfection analysis and reporter constructs.

Transcriptional activity of receptor variants was assessed by measuring their effect on the activity of chloramphenicol acetyltransferase (CAT) or luciferase (Luc) reporter genes expressed in HeLa cells. For transient transfection experiments, we used the calcium phosphate co-precipitation technique, as detailed in previous reports. Plasmids expressing wild type or variant ER-α cDNAs were transfected into recipient cells along with one of a variety of estrogen-responsive reporter plasmids (see below). Typically, 1.5 X 10^6 cells were plated one day prior to transfection in 60 mm dishes and were treated with 0.5 μg of receptor expression plasmid and 10 μg of reporter plasmid. Total input DNA was normalized using an empty pCMV-4 vector, which also served as a negative control. Transfected cells were cultured for 48 hours in DMEM containing 5% charcoal-treated calf serum, in the absence and presence of 5 nM estradiol or 20 nM phorbol 12-myristate 13-acetate (PMA). For each experiment, CAT and Luc activities (corrected for protein concentration) were normalized to the basal level of expression seen for untreated pCMV-4 control transfections. The estrogen-responsive reporter plasmids used for these experiments included pERE-TK-CAT (6), pOvalb-Cat obtained from M. Sanders (7), pAP-1_3TK-CAT kindly provided

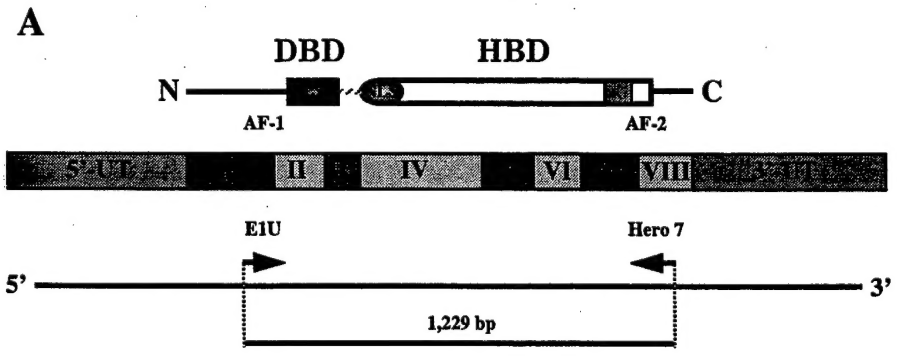
by L. McCabe (Dept. of Physiology, MSU), Coll73-Luc received from P. Kushner (8), and pIGF(1630)-Luc and pIGF(926)-Luc obtained from P. Rotwein (9).

RESULTS

Clonal analysis of variant ER-α transcripts in MCF10AT derivatives, TG-1 and TG-3c cells.

Following an approach previously described for the semiquantitative analysis of ER-α splicing variants in MCF-7 cells (see year 3 technical report), ER-α mRNA transcripts were similary characterized in derivatives of the MCF10AT cell line, TG-1 and TG-3c. MCF10A cells represent a spontaneously immortalized (but non-tumorigenic) human mammary epithelial cell line established at the Karmanos Cancer Center (Detroit, MI) from a woman with benign fibrocystic breast disease (10). This woman remains in good health and is cancer-free. These cells were stably transfected with T24-Ha*Ras* to give rise to the MCF10AT cell line. When carried as (subcutaneous) xenografts in nude mice, MCF10AT cells give rise to histologically heterogeneous lesions with components that closely resemble human atypical hyperplasia, ductal carcinoma *in situ*, and invasive adenocarcinoma (11). As such, MCF10AT cells appear to represent a useful model for preneoplastic human breast disease whose progression can be followed in nude mice xenografts. The TG-1 and TG-3c sublines represent subcultures from first and third cycle of serial xenografts re-established from lesions that histologically resembled squamous carcinoma and carcinoma *in situ*, respectively. Like the parental MCF10AT cells, TG-1 and TG-3c cells also give rise to highly heterogeneous preneoplastic lesions in nude mice, including atypical hyperplasia. However, they appear to progress towards DCIS and invasive carcinoma with shorter latency and higher frequency than the MCF10AT line (12).

While the parental MCF10A cell line expresses such low levels of ER-α as to be essentially ER-negative, the *Ras*-transformed MCF10AT cells, as well as their xenograft derivatives TG-1 and TG-3c, are demonstrably positive with respect to expression of ER-α mRNA (11,12). Additionally, there is evidence that TG-3c cells are estrogen-responsive based on their increased cloning efficiency and larger colony size when cultured in soft agar in the presence of estradiol (11,12) and based on their more rapid progression to a neoplastic state when xenografted into animals receiving an estradiol implant (12). For these reasons, it was felt that a more detailed analysis of ER-α mRNA transcripts in TG-1 and TG-3c cells was justified. To do so, an RT/PCR approach was taken, followed by an extensive clonal analysis of the ER-α amplification products. As depicted in Fig. 1A, the PCR primers chosen for this analysis were targeted to exons I and VIII to permit a thorough analysis of the complete profile of known ER-α splicing variants. Fig. 1B shows that the PCR amplification products from both TG-1 and TG-3c cells are heterogeneous in size, and include full-length transcripts (1,229 bp) as well as discrete products shorter in size.



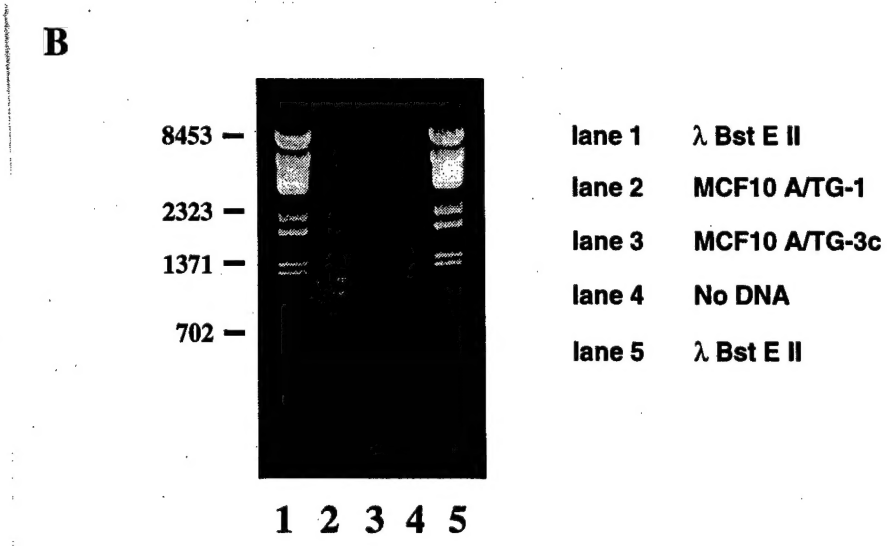


Fig. 1. RT/PCR amplification of ERα mRNAs present in MCF10A/TG-1 and TG-3c cells: A) design of the amplification experiment showing the locations of PCR primers, B) EtBr-stained agarose gel showing the size distribution of PCR products.

Products from these TG-1 and TG-3c amplification reactions were cloned without further purification into the pCR2-TOP cloning vector (InVitrogen, Inc.) and were analyzed as previously described for MCF-7 cDNA clones (see year 3 annual technical report). Approximately 100 white (insert-containing) colonies were picked for each cell line to enable characterization of their inserts and to confirm their identity as authentic ER-α cDNA clones. Clones were assigned using a battery of restriction digests and cDNAs representative of each insert size class were sequenced to confirm that they were correctly assigned. Data from this analysis are summarized in Fig. 2. While the profile of ER-α splicing variants that we observe in both TG-1 and TG-3c cells is reminiscent of the extensive transcript heterogeneity that we previously reported for the MCF-7 cell line (see year 3 annual technical report), two significant differences can be noted. In MCF-7 cells, correctly processed ER-α mRNA (hereafter referred to as wild type receptor, or wt ER-α) was easily the most abundant transcript observed, representing nearly 50% of the clones analyzed. In contrast, in both TG-1 and TG-3c cells, wt ER-α cDNAs were equaled or exceeded in abundance by four discrete splicing variants (ERΔE2, ERΔE3, ERΔE4, & ERΔE7). Additionally, clones isolated from TG-1 and TG-3c cells included only "simple" exon-skipped variants, although 15-20% of these variants harbored the loss of more than a single exon. A much more complex situation was observed in MCF-7 cells where approximately 10% of all the clones analyzed contained internal deletions that failed to mapped to known exon boundaries and therefore appeared to arise from the use of cryptic splice donor or acceptor sites (see year 3 annual technical report). Such cryptic splice products were not observed in either TG-1 or TG-3c cells. Our results confirm that both MCF10A/TG-1 and TG-3c cells appear to be ER-α positive and to express, at a fairly significant level, a wide array of splicing variants in addition to wt ER-α mRNA. Corroboration of these results at the protein level is underway and will involve immunoblot analysis using a battery of ER-α specific antibodies with non-overlapping epitopes. It is uncertain, however, if analysis at the protein level will give a clear picture due to the heterogeneity of protein variants predicted to occur based on the complexity of RNA variants that we observe.

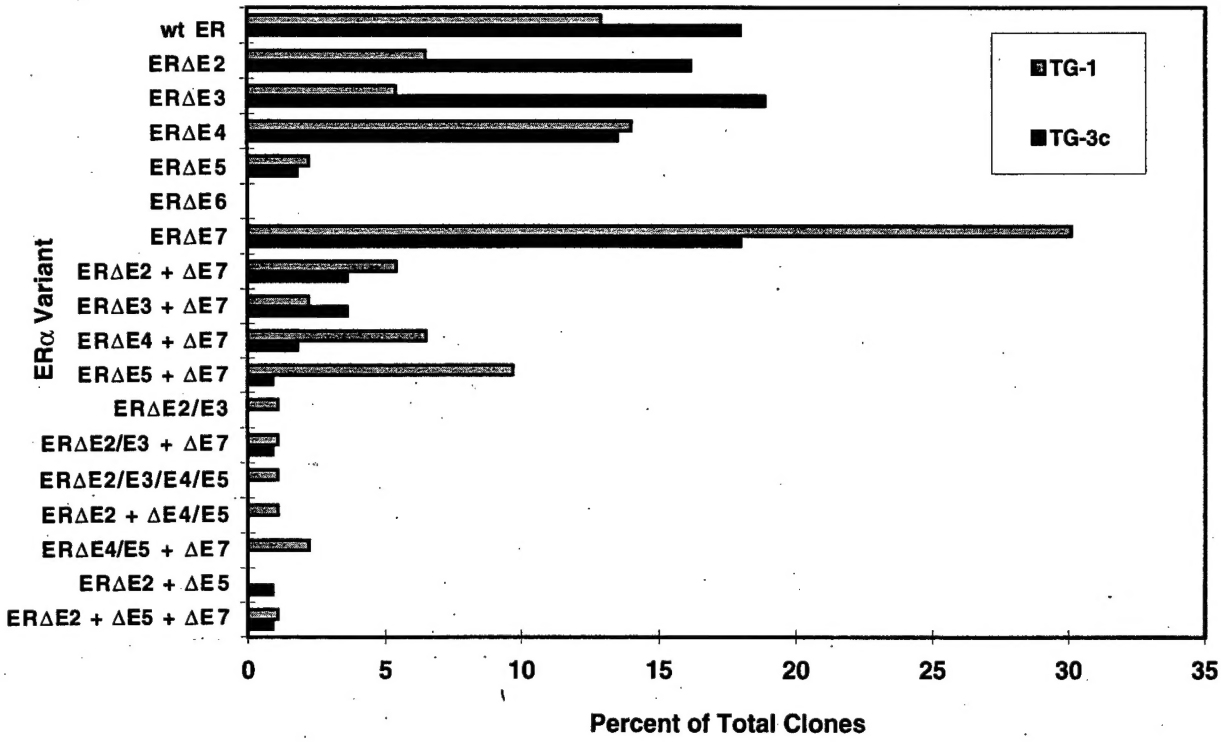


Fig. 2. Quantitative summary of the ERα cDNA clones from MCF10A/TG-1 and TG-3c cells. Clones were isolated from the PCR amplification experiment shown in Fig. 1B and characterized by restriction analysis and DNA sequencing. The numbers depicted indicate the percentage of total clones from the analyzed pool that were represented by each individual ERα splicing variant. A total of 111 ERα cDNAs were characterized for the TG-1 cell line and 93 cDNAs were characterized from TG-3c cells.

Analysis of variant ER-α transcripts in M13 SV1 cells.

As an additional model for ER-α expression in cells derived from the normal human breast, we chose to examine several cell lines developed in the laboratory of C.C. Chang (Dept. of Pediatrics, MSU). These cells originated from a reduction mammoplasty and represent a cell population (referred to as Type I cells) that display luminal and stem cell characteristics (13). When grown in Matrigel together with differentiated Type II breast epithelial cells, Type I cells form organoids that assemble into branching ducts that are capped by structures resembling terminal endbuds. Type I cells were immortalized by treatment with SV40 virus to give rise to a series of clones including M13/SV1, which are immortal but non-tumorigenic in nude mice (Fig. 3). The immortal cells were subsequently X-Ray irradiated and transformed with cErbB/Neu, giving rise to the tumorigenic M13/R_2N_1 cell line. Cells from this series express keratin 18, but not keratin 14 or α6 integrin. Based on immunoblot and preliminary RT/PCR analysis both M13/SV1 and M13/R_2N_1 cells were shown to be ER-positive, but were reported to express a size variant of ER-α (14). For this reason, we chose to perform additional PCR analysis of ER-α transcripts in this cell line.

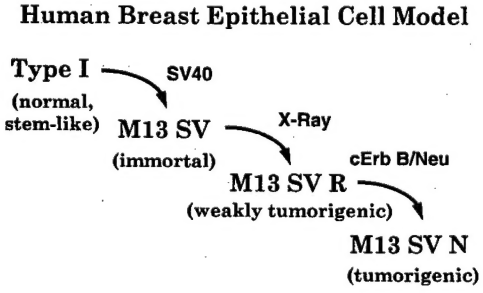


Fig. 3. Origin of M13 SV1 and M13 R2N1 breast epithelial cells

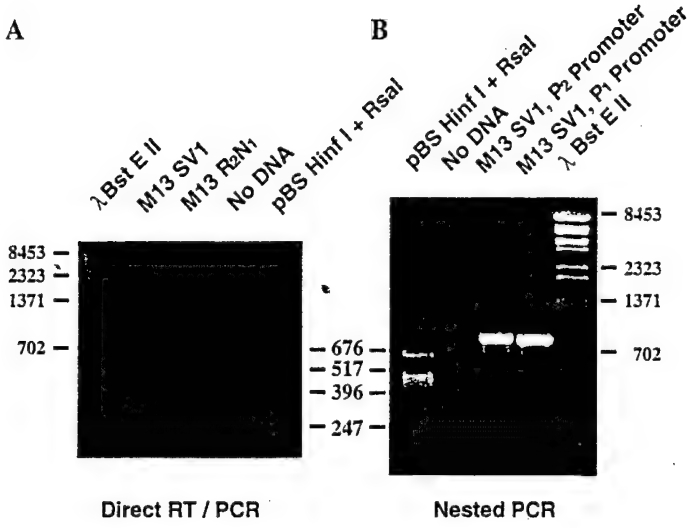


Fig. 4. Example of an ERα PCR experiment in M13/SV1 and M13/R2N1 cells, showing the heterogeneity in ERα transcript sizes encompassing exons 1 through 5. The panel on the left represents a direct RT/PCR experiment, while the panel on the right is from a nested PCR experiment using promoter-specific primers for the first round of PCR.

PCR amplification was performed as described above for MCF10A/TG-1 and TG-3c cells, using a variety of ER-α primer pairs. An example of one such experiment is given in Fig. 4, representing amplification of an 848 bp interval between exons 1 and 5. The majority of product corresponds to a band of the expected size, with a number of less intense bands smaller in size. The heterogeneity of PCR products is very reminiscent of what we observe in other ER-positive cell lines, such as MCF-7, and is consistent with species including ERΔE2 (657 bp), ERΔE3 (731 bp), ERΔE4 (513 bp), and ERΔE2/E3 (540 bp). Equivalent results were obtained with primer pairs encompassing the entire protein coding region (data not shown). As shown by a nested PCR experiment in which an initial round of amplification used P_1 or P_2-specific primers (from the 5'-untranslated regions) paired with an exon 8 lower stranded primer followed by reamplification with the exon 1/exon 5 primer pair, ER-α transcripts present in the M13 SV1 cell line originate from both the proximal (P_1) and distal (P_2) promoters. These promoters give rise to mRNAs with alternative 5'-untranslated exons that are spliced onto the body of the ER-α protein coding region. Due to the similarity between the pattern of ER-α transcripts in M13/SV1 and M13/R_2N_1 cells with what we previously observed in MCF-7 and MCF10A/TG-1 and TG-3c cells, further cloning and characterization of these PCR products was not performed. Our RT/PCR analysis of ER-α mRNA in cells of the M13 series therefore confirm previous conclusions regarding their ER-positive status, but failed to detect the presence of a predominant ER-α size variant as suggested by Kang, et al. (14). Rather, like other ER-positive cell lines, these cells appear to express predominantly wt ER-α along with a heterogeneous population of smaller ER-α variants.

Characterization of the functional activity of ER splicing variants on "non-classical" estrogen responsive promoters.

We have previously reported that, while the ERΔE5 splice variant may display modest transcriptional activity in some special contexts, it is largely devoid of transcriptional stimulatory effects that require it to bind directly to a palindromic estrogen response element (ERE). This is entirely consistent with the dramatically reduced DNA-binding affinity relative to wt ER-α that we observe in gel mobility shift experiments. Similarly, the remaining exon-skipped ER-α splicing variants (ERΔE2, ERΔE3, ERΔE4, ERΔE6, & ERΔE7) appear to completely lack positive

transcriptional activity when tested in transient transfection assays with a pERE-TK-CAT reporter plasmid (see year 3 annual technical report). Indeed, we have concluded that the most prominent activity of two of the splicing variants (namely ERΔE3 and ERΔE5) on ERE-containing genes is to interfere with the transcriptional stimulatory activity of wt ER-α, acting as dominant-negative mutants with modest potency. The inhibitory activity of ERΔE3 and ERΔE5 is likely to result from their ability to form functionally inactive, mixed dimers with intact ER-α, to sequester steroid receptor coactivators or other limiting basal transcription factors into non-productive complexes, or by a combination of both mechanisms.

These results, however, leave open the possibility that some of the ER-α splicing variants may display transcriptional stimulatory effects on the growing list of estrogen responsive genes that lack a canonical ERE. For these genes, transcriptional stimulation by ER appears to be mediated by an indirect mechanism, involving synergistic protein-protein interactions with upstream transcription factors (such as AP-1 or SP-1) or nuclear receptor coactivators (including CBP/p300, SRC-1, TIF-2, GRIP1, or AIB-1). To further examine this possibility, we have tested the effect of co-transfecting individual ER-α splice variants, compared with an empty expression vector, on the activity of four reporter plasmids that were selected from the literature based on their ability to be transcriptionally induced by ER, despite their apparent lack of a canonical ERE. These plasmids included $p(AP1)_3$TK-CAT (driven by three consensus AP-1 sites upstream of the HSV thymidine kinase promoter), Coll73-Luc (driven by the -73/+63 human collagenase promoter), pIGF1630-Luc (driven by the -1630/+322 human IGF-1 promoter), and pOvalb-CAT (driven by a 1350 bp promoter fragment from the chicken ovalbumin gene). Data from experiments in which each of the ER-α splicing variants were individually tested on these four reporter constructs are summarized in Figs. 5-8. For screening purposes, HeLa cells were chosen as the host for transfection with each one of the reporters together with expression plasmids for wt ER-α, one of the splicing variants, or the empty pCVM-4 expression vector. Paired cultures were treated with 10 nM 17β-estradiol (E) in combination with 20 nM phorbol 12-myristate 13-acetate (PMA), or with vehicle alone. This was done to ensure that an activating ligand (E) was present, if necessary, and that AP-1 was maximally stimulated in the event that promoter activation involved an AP-1 element. Of the reporter constructs that we examined, only pOvalb-CAT was consistently and significantly induced by wt ER-α; however, all four reporter constructs displayed a reproducible transcriptional response to one or more of the ER-α splicing variants (either ERΔE3 or ERΔE5). These findings are detailed below.

As previously reported by Gaub et al. (15) and confirmed by the experiment shown in Fig. 5, efficient stimulation of ovalbumin transcription by wt ER-α in transfected HeLa cells requires both the presence of estradiol and activation of AP-1 by phorbol ester. A modest, but significant stimulation of this reporter is also observed in response to PMA treatment alone, but estradiol by itself is essentially without effect. Thus, estrogen regulation of the non-canonical hormone response element within the ovalbumin reporter involves strong transcriptional synergism between ER-α and AP-1. Mechanistically, this phenomenon is thought to involve a protein-protein interaction between ER-α and AP-1 (Jun/Fos heterodimers), acting through a consensus TPA response element (also known as a TRE, or an AP-1 site). Whether this involves a direct interaction between ER-α and the AP-1 components Jun and Fos, or an indirect interaction mediated through a bridging factor such as CBP/p300 or SRC-1 still needs to be clarified. Interestingly, while the chicken ovalbumin promoter contains a consensus AP-1 site, it lacks a palindromic ERE and doesn't appear to require the direct binding of ER-α to DNA. This finding was previously reported by Gaub and coworkers (15) who used genetically engineered receptor mutants to establish that the integrity of its DNA-binding domain was *not* required for ER-α to activate the AP-1 element within the ovalbumin promoter. Consistent with this fact, we observe that a *naturally* occurring ER-α splice variant (ERΔE3) that contains an in-frame deletion within its DNA-binding domain is approximately 40% as active as wt ER-α on this reporter construct.

Similar to the wt receptor, the activity of ERΔE3 requires the combination of estradiol and a phorbol ester. Among the other ER-α splicing variants tested with the pOvalb-CAT reporter (ERΔE2 through ERΔE7, data not shown), only the ERΔE5 variant displayed some stimulatory activity. While the effect of ERΔE5 was modest, this 3-fold stimulation (compared with vector controls) was reproducible and statistically significant. Since ERΔE5 lacks an intact ligand-binding domain, its activity was unaffected by estradiol, but remained dependent upon treatment of cells with phorbol ester.

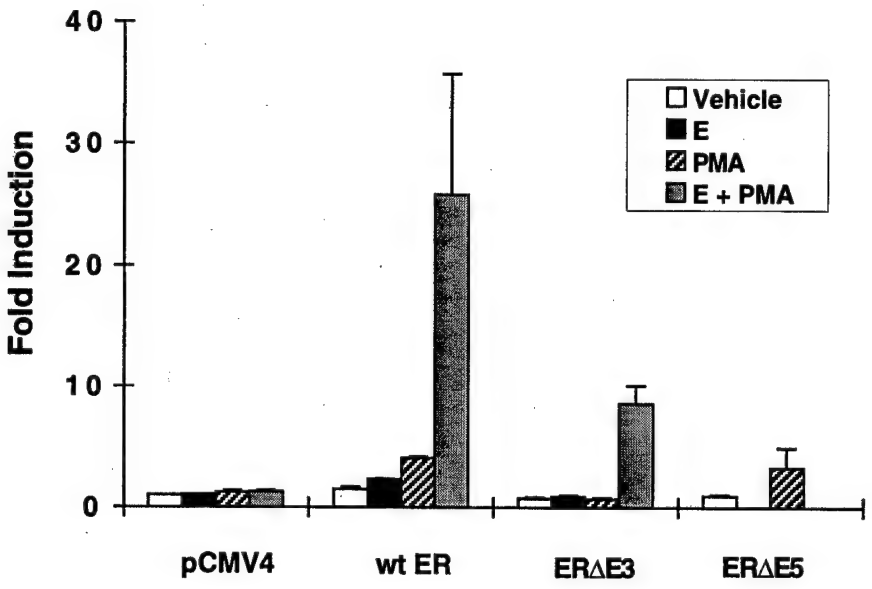


Fig. 5. Transcriptional stimulatory activity of wt ERα, ERΔE3, and ERΔE5 on a co-transfected pOvalb-CAT reporter plasmid. HeLa cells were transiently co-transfected with 10 ug of reporter and 0.5 ug of the indicated ER expression vector and were treated as indicated: vehicle control, 20 nM phorbol 12-myristate 13-acetate (PMA), 10 nM estradiol (E), or both (E + PMA). CAT assays were normalized for protein and values are expressed relative to vehicle-treated empty expression vector (pCMV-4) controls. Error bars represent the standard error of the mean of three independent experiments.

The Coll73-Luc (human collagenase) reporter represents a second well characterized AP-1 responsive reporter plasmid (8) that gave generally similar results with co-transfected ER-α splicing variants (see Fig. 6). Compared with the pCMV-4 vector control transfection which measures induction due to endogenous AP-1 in the absence of ER-α, the ERΔE3 variant supported a 6- to 10-fold higher stimulation of Coll73-Luc expression in the combined presence of estradiol and phorbol ester. To a lesser extent, the ERΔE5 variant also stimulated collagenase promoter activity. The stimulatory effect of ERΔE5 was observed in both treated, as well as untreated cells, with phorbol ester producing an approximate doubling of the transcriptional response. The remaining splicing variants (ERΔE2, ERΔE4, ERΔE6, and ERΔE7) were indistinguishable from the empty vector control (pCMV-4). Somewhat unexpectedly, wt ER-α is largely without effect on the Coll73-Luc reporter construct in our hands (Fig. 6). Webb and coworkers (16) have described that intact ER-α in the presence of estrogen elicits a very modest (~2-fold) stimulation of the Coll73-Luc reporter in HeLa cells. These workers describe other conditions where a more robust response can be achieved. These include the use of collagenase promoter fragments containing additional upstream sequences (16), treatment with the antiestrogen tamoxifen instead of estradiol (16), or the use of agonist-liganded ER-β as opposed to ER-α (17). The apparent difference in the behavior of wt ER-α on this reporter construct are still under investigation. Our results with the Coll73-Luc reporter are nonetheless significant in that they indicate that two of the ER-α splicing variants (ERΔE3 and ERΔE5) actually appear to be more potent inducers of the

human collagenase reporter than the full-length ER-α protein. Further experiments are planned to separately characterize the need for ligand and phorbol ester to activate the ER-α variants on the collagenase reporter and to compare the activity of tamoxifen with estradiol.

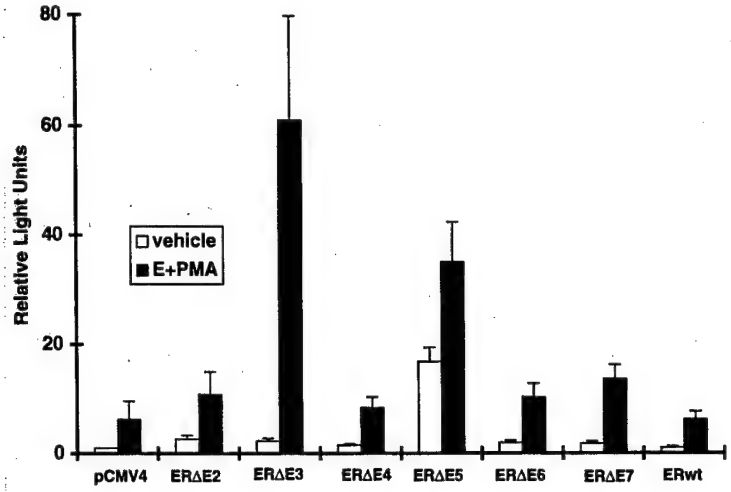


Fig. 6. Transcriptional stimulatory activity of ERα splicing variants on a co-transfected human collagenase reporter plasmid, Coll73-Luc. HeLa cells were transiently transfected as described in the legend to Fig. 5. Luciferase activities were normalized for protein and results are expressed relative to vehicle-treated empty expression vector (pCMV-4) controls. Error bars represent the standard error of the mean of three independent experiments.

A slightly different response pattern was observed when the ER-α splicing variants were tested with $p(AP1)_3$TK-CAT, an engineered reporter plasmid containing three consensus AP-1 elements upstream of the thymidine kinase reporter. As shown in Fig. 7, ERΔE5 was unique among the receptor constructs examined in its ability to induce the $p(AP1)_3$TK-CAT reporter in HeLa cells. In this case, it is significant that both wt ER-α and ERΔE3 *fail* to induce this reporter construct when treated with estradiol and phorbol ester. Indeed, there are subtle indications that wt ER-α and ERΔE3 may actually be inhibitory in this context although this inhibition does not reach statistical significance. Again, while there is a widespread impression that wt ER-α is able to act as an estrogen-dependent inducer of transcription through many AP-1 response elements, the data presented here are actually in good agreement with published reports using analogous reporter constructs.

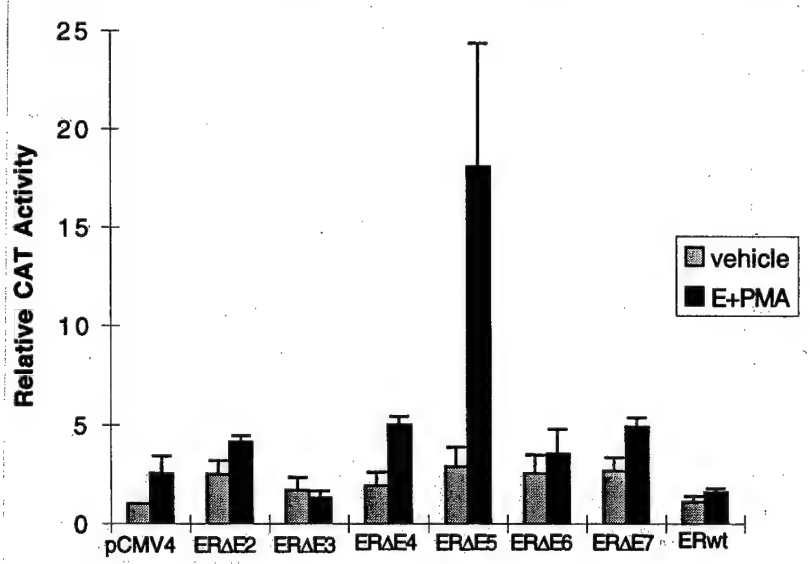


Fig. 7. Transcriptional stimulatory activity of ERα splicing variants on a reporter plasmid, $p(AP1)_3$TK-CAT, that contains three consecutive AP-1 elements upstream of the thymidine kinase promoter. HeLa cells were transiently transfected as described in the legend to Fig. 5, above. CAT activities were normalized for protein and values are expressed relative to vehicle-treated empty expression vector (pCMV-4) controls. Error bars represent the standard error of the mean of three independent experiments.

Specifically, Webb and coworkers (16) have shown that the collagenase AP-1 site, multimerized upstream of the HSV tk promoter (similar to the p(AP1)$_3$TK-CAT reporter used in our studies) displayed a modest (~2-fold) transcriptional response to antiestrogens (tamoxifen and ICI-164,384), but *not* to estradiol, when co-transfected with wt ER-α. We therefore feel that the 5-fold stimulation of CAT expression that we observe through a consensus AP-1 element (TGAGTCA) in response to the naturally occurring ER-α splicing variant (ERΔE5) represents a highly significant finding. Preliminary experiments to dissect the requirements for this induction indicate that it depends upon treatment of transfected cells with phorbol esters and that, as expected for a variant that lacks an intact ligand-binding domain, it is unaffected by estradiol (data not shown). Further experiments to clarify this point and to assess the involvement of nuclear receptor co-activators in this phenomenon are underway.

The IGF-1 gene is expressed in a variety of tissues, including the liver, bone, uterus and breast, where it contributes to the normal growth and differentiation of these structures (18). Some uncertainty remains regarding the sources of IGF-1 within the breast and with respect to its role in breast tumor growth. It is likely that the mammary epithelium is exposed to IGF-1 from systemic sources as well as from the breast stroma. In addition, some breast tumors produce IGF-1 and other growth factors of their own (19). Expression of IGF-1 (and other growth factors) appears to be deregulated in some tumors, contributing an autocrine component to tumor cell growth. The potential of ER-α splicing variants to stimulate transcription of the IGF-1 gene in breast tumor cells is therefore of potential clinical significance. Interestingly, we observe that ERΔE5, but not ERΔE3 or wt ER-α promotes expression of a co-transfected IGF-1 luciferase reporter plasmid (IGF1630Luc) in HeLa cells (Fig. 8). This stimulation is preserved on IGF-1 promoter fragments containing as little as 592 bp of upstream sequence (data not shown). As with the behavior of the p(AP1)$_3$TK-CAT reporter shown above, the activity of ERΔE5 implicates the N-terminal activation domain (AF1) or the DNA-binding domain in this effect, while the inactivity of wt ER-α and ERΔE3 suggests that presence of the C-terminal activation domain (AF2) prevents ER-α from stimulating IGF-1 expression. However, in contrast with the p(AP1)$_3$TK-CAT reporter, the failure of phorbol ester to stimulate the activity of ERΔE5 on the IGF-1 promoter suggests that the AP-1 factor is unlikely to be involved and that transcription factors other than jun or fos must represent the targets for ERΔE5 interaction. Experiments are in progress to map the cis element within the IGF-1 promoter that is responsible for regulation by ERΔE5.

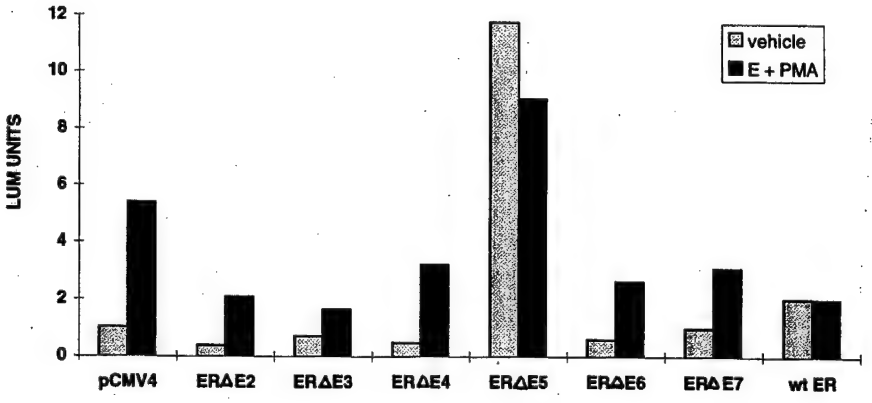


Fig. 8. Transcriptional stimulatory activity of ERα splicing variants on a co-transfected human IGF-1 reporter plasmid, pIGF(-1630)-Luc. HeLa cells were transiently transfected as described in the legend to Fig. 5, above. Luciferase activities were normalized for protein and values are expressed relative to vehicle-treated empty expression vector (pCMV-4) controls. Error bars represent the standard error of the mean of three independent experiments.

DISCUSSION

The major accomplishments for this reporting period include: 1) a thorough analysis of ER-α variants in TG-1 and TG-3c cells, two xenograft lines derived from the MCF10AT line, 2) the use of RT/PCR for a preliminary analysis of ER-α variants in M13 SV1 and R_2N_1 cells, 3) and continued characterization of the functional activity of two key ER-α splicing variants. The first two projects fall broadly within Task 1.d of our original statement of work, whose goal has been to clarify the status of ER-α splicing variants in normal breast epithelial cells. Since the inception of this project, several careful studies have been reported in the literature comparing ER-α mRNA species between breast tumors and normal human breast tissue (1,3). Based on these and earlier studies, a widespread consensus has emerged that ER-α splicing variants are not unique to the neoplastic breast, but appear to coexist with "correctly" processed ER-α mRNA in essentially all estrogen-responsive tissues and tumors that have so far examined. Rather than reproducing these published studies, we chose a slight redirection of our efforts to characterize ER-α transcripts in two fairly recently developed ER-positive mammary cell culture systems (derivatives of MCF10A and Type I M13 cells) that are being proposed as models for transformation and malignant progression in the normal human breast.

Work during this reporting period served to confirm and extend our previous conclusions by showing that ER-α transcripts in the MCF10A/TG-1, TG-3c, M13 SV1, and R_2N_1 cells are remarkably heterogeneous in nature, similar to MCF-7 and T47D breast tumor cells that we analyzed previously. In the majority of these cell lines, correctly processed ER-α mRNA is the single most abundant ER-α species observed. This is apparently *not* the case in MCF10A/TG1 and TG-3c cells, where the ERΔE7 variant equals or exceeds wt ER-α in abundance (Fig 2). It is important to note, however, that in the aggregate ER-α splicing variants represent a significant fraction (usually ≥ 50%) of the total ER-α mRNA pool. Difficulty in documenting the existence of these variants at the protein level is often cited as evidence that ER-α variants may be unstable proteins that are of minor importance for estrogen signalling. The most likely reason for this is the finding that individually, most variants represent less that 5% of the structurally diverse pool of ER-α transcripts and are therefore predicted to give rise to an equally diverse population of low abundance variant ER-α proteins. While immunological detection techniques are generally suitable for detecting full length (67 kd) ER-α (which is usually the most abundant species), they are probably not sensitive enough to detect the lower abundance splicing variants. An ER-α species in the 48-52 kd size range reactive with many ER antibodies is observed by most investigators; however, it is generally dismissed as a probable proteolytic product. An alternative interpretation is that this band corresponds to the 52 kd ERΔE7 variant that is consistently found to be the most abundant of the variant ER-α RNAs. In an effort to clarify this situation, studies are underway during the final award year to analyze cross-reacting ER-α proteins in these ER-positive cell lines using a battery of monoclonal antibodies that recognize defined epitopes within the ER-α polypeptide chain. In parallel, transfection studies using a variety of estrogen-responsive reporter plasmids are being conducted in the MCF10A/TG-1, TG-3c, M13 SV1, and R_2N_1 cell lines to assess their estrogen responsiveness.

The other major activity for this reporting period involved additional analysis of the transcriptional activity of ER-α splicing variants on promoters containing non-canonical estrogen response elements (Task 2.b). The traditional view of estrogen action depicts ER-α as the central player in target gene selection by virtue of its ability to bind to DNA containing a consensus estrogen response element (ERE). More recent evidence indicates that many estrogen regulated genes contain non-canonical hormone response elements organized around AP-1 or Sp1 sites (8,15). Among these targets are genes such as IGF-1, collagenase, cathepsin D, cyclin D1, c-fos, c-myc, and RARα that are believed to play important roles both during normal breast development

and during tumor formation and progression. Of interest, the structural requirements for ER-α to regulate many non-canonical hormone response elements are less stringent than transcriptional stimulation through a consensus ERE. For this reason, we have examined the behavior of ER-α splicing variants on four reporter constructs containing non-canonical hormone response elements. In agreement with published reports, wt ER-α stimulated ovalbumin promoter activity. The dual requirement for estradiol and a phorbol ester indicate that this regulation occurs through an AP-1 site identified in the upstream region of the ovalbumin promoter (15) and involves a cooperative interaction between ER-α and the Jun/Fos heterodimer. A significant estradiol- and phorbol ester-dependent induction of ovalbumin activity was also supported by ERΔE3, despite its lack of an intact DNA-binding domain. ERΔE5 also supported a modest stimulation of ovalbumin activity that was phorbol ester-dependent, but estradiol-independent. These observations indicate that stimulation of the ovalbumin promoter by ER-α is mechanistically complex and includes both a phorbol ester-sensitive pathway involving the amino terminal activation domain (AF1) as well as an estradiol-sensitive pathway involving the carboxy terminal activation domain (AF2). While the activities of ERΔE3 and ERΔE5 imply that direct DNA binding by ERΔE5 is not involved in regulation of ovalbumin gene expression, it is clear that neither of these splicing variants is as active as wt ER-α on this promoter construct.

In contrast to the situation with pOvalb-CAT, the activity of wt ER-α on the remaining three non-canonical hormone response elements was insignificant compared to the that of the ERΔE3 or ERΔE5 splicing variants. Specifically, both ERΔE3 and ERΔE5 were active on the Coll73-Luc reporter, while only ERΔE5 was active on the pIGF(-1630)-Luc and $p(AP1)_3$TK-CAT reporter constructs. In the case of the collagenase promoter fragment, induction by ERΔE3 required both estradiol and phorbol ester treatment. The stimulatory activity of ERΔE5 required phorbol ester treatment for the $p(AP1)_3$TK-CAT reporter, was modestly enhanced by phorbol ester on the Coll73-Luc reporter, and was independent of phorbol ester treatment on the pIGF(-1630)-Luc reporter. This would appear to suggest that activation of AP-1, presumably by stimulation of protein kinase C and phosphorylation of Jun, plays different roles in the stimulation of these various reporter constructs by ERΔE5. These data also suggest that the presence of a functional AF2 domain is detrimental for activation of some (but not all) non-canonical hormone response elements by ER-α since ERΔE5, but not wt ER-α or ERΔE3 stimulated the $p(AP1)_3$TK-CAT and pIGF(-1630)-Luc reporter plasmids. Our observations provide the first solid evidence that two ER-α splicing variants, namely ERΔE3 and ERΔE5, like wt ER-α can exert significant positive effects on gene transcription and raise the intriguing possibility that each one of these receptor isoforms may target a unique, but overlapping subset of genes for transcriptional regulation. In the final year of this project, our efforts will focus on understanding the mechanism(s) of gene regulation by ER-α splicing variants.

Publications From This Award

1) year 4 publication related to this award (copies included as an Appendix)

   a) Bollig, A. and Miksicek, R.J. (1999) An estrogen receptor-α splicing variant mediates both positive and negative effects on gene transcription. *Mol. Endocrinol.*, submitted January 1999.

2) manuscripts currently in preparation from this award

   a) Ankrapp, D.P., Morrison, M., Bollig, A.B., and Miksicek, R.J. MCF-7 and MCF10AT breast epithelial cells express a broad array of ERα mRNA size variants, in preparation.

3) previous publications related to this award

   a) Bunone, G., Briand, P.-A., Miksicek, R.J., and Picard, D. (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.*, 15(9):2174-2183.
   b) Elias, J.M., Hyder, D.M., Miksicek, R.J., Heimann, A., and Margiotta, M. (1995) Interpretation of steroid receptors in breast cancer: a case with discordant estrogen receptor results using ER1D5 and H222. *J. Histotechnology*, 18(4):331-335.

4) current publications unrelated to this award

   a) Miksicek, R.J., Lee, C., and Morrison, M. Synthetic peptides derived from dimerization motifs within estrogen receptor-α interfere with receptor DNA-binding, in preparation.
   b) Wang, S.F., Miura, K., Miksicek, R.J., Segraves, W.A., and Raikhel, A.S. (1998) DNA binding and transactivation characteristics of the mosquito ecdysone receptor-ultraspiracle complex. *J. Biol. Chem.*, 273:27531-27540.

REFERENCES

1) Gotteland, M., Desauty, G., Delarue, J.C., Liu, L., and May, E. (1995) Human estrogen receptor messenger RNA variants in both normal and tumor breast tissues. *Mol. Cell. Endocrinol.* **112**:1-13.
2) Murphy, L.C., Leygue, E., Dotzlaw, H., Douglas, D., Coutts, A., and Watson, P.H. (1997) Oestrogen receptor variants and mutations in human breast cancer. *Trends in Mol. Med.* **29**:221-234.
3) Leygue, E., Watson, P.H., and Murphy, L.C. (1996) Estrogen receptor variants in normal human mammary tissue. *J. Nat. Cancer Inst.* **88**:284-290.
4) Fuqua, S.A.W., Chamness, G.C. and McGuire, W.L. (1993) Estrogen receptor mutations in breast cancer. *J. Cell. Biochem.* **51**:135-139.
5) Fuqua, S.A.W, Wolf, D.M. (1995) Molecular aspects of estrogen receptor variants in breast cancer. *Breast Cancer Res. and Treatment*, **35**:233-241.
6) Klock, G., Strähle, U., and Schütz, G (1987) Oestrogen and glucocorticoid responsive elements are closely related but distinct. *Nature* **329**:734-736.
7) Sanders, M.M. and McKnight, G.S. (1988) Positive and negative regulatory elements control the steroid-responsive ovalbumin promoter. *Biochem.* **27**:6550-6557.
8) Uht, R.M., Anderson, C.M., Webb, P., and Kushner, P.J. (1997) Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element. *Endocrinol.* **138**:2900-2908.
9) Kim, S.W., Lajara, R., and Rotwein, P. (1991) Structure and function of a human insulin-like growth factor-I gene promoter. *Mol. Endocrinol.* **5**:1964-72.
10) Soule, H.D., Maloney, T.M., Tait, L., Heppner, G.H., and Miller, F.R. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF10. *Cancer Res.* **50**:6075-6086.
11) Shekhar, P.V.M., Werdell, J., and Basrur, V.S. (1997) Environmental estrogen stimulation of growth and estrogen receptor funtion in preneoplastic and cancerous human breast cell lines. *Int. J. Cancer* **89**:1774-1782.
12) Shekhar, M.P., Nangia-Makker, P., Wolman, S.R., Tait, L., Heppner, G.H., and Visscher, D.W. (1998) Direct action of estrogen on sequence of progression of human preneoplastic breast disease. *Am. J. Pathol.* **152**:1129-1132.
13) Kao, C.Y., Nomata, K. Oakley, C.S., Welsch, C.W., and Chang, C.C. (1995) Two types of normal human breast epithelial cells derived from reduction mammoplasty: phenotypic characterization and response to SV40 transfection. *Carcinogenesis* **16**:531-538.
14) Kang, K.S., Morita, I., Cruz, A., Jeon, Y.J., Trosko, J.E., and Chang, C.C. (1997) Expression of estrogen receptors in a normal human breast epithelial cell type with luminal and stem cell characteristics and its neoplastically transformed cell lines. *Carcinogenesis* **18**:251-257.
15) Gaub, M.-P., Bellard, M., Scheuer, I., Chambon, P., and Sassoni-Corsi, P. (1990) Activation of the ovalbulmin gene by the estrogen receptor involves the Fos-Jun complex. *Cell* **63**:1267-1276.
16) Webb, P., Lopez, G.N., Uht, R.M., and Kushner, P.J. (1995) Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Mol. Endocrinol.* **9**:443-456.
17) Paech, K., Webb, P., Kuiper, G.G.J.M., Nilsson, S., Gustafsson, J.-A., Kushner, P.J., and Scanlan, T.S. (1997) Differential ligand activation of estrogen receptors ER-α and ER-β at AP1 sites. *Science* **277**:1508-1510.
18) Stewart, C.E. and Rotwein, P. (1996) Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol. Rev.* **76**:1005-26.
19) Pilihowska, M., Kimura, N., Fujiwara, H., and Nagura, H. (1997) Immunohistochemical study of TGF-alpha, TGF-beta1, EGFR, and IGF-1 expression in human breast carcinoma. *Mod. Pathol.* **10**:9699-9675.

An Estrogen Receptor-α Splicing Variant Mediates Both Positive and Negative Effects on Gene Transcription

Aliccia Bollig and Richard J. Miksicek

Michigan State University
Department of Physiology
East Lansing, MI 48824-1101

Corresponding Author: Richard J. Miksicek
Department of Physiology
108 Giltner Hall
Michigan State University
East Lansing, MI 48824-1101

Phone: (517)-355-6475 x 1285
Fax: (517)-355-5125
email: miksicek@psl.msu.edu

Keywords: Steroid Receptors, Coactivators, SRC-1, AP-1, Ovalbumin

ABSTRACT

Analysis of messenger RNA (mRNA) prepared from a variety of estrogen-responsive cell lines, breast tumor specimens, and normal breast tissue have established that estrogen receptor-α (ERα) mRNA is typically expressed as a mixture of transcripts. Using PCR amplification, this heterogeneity has been shown to result largely from an imprecise pattern of mRNA splicing that gives rise to a family of correctly processed and exon-skipped ERα transcripts. We have reconstructed ERα cDNAs representing the single exon-skipped variants ERΔE2 through ERΔE7 to enable their functional characterization in a well defined cell transfection system. All six of the ERα splicing variants can be efficiently and stably expressed in Cos7 cells, and each displays a characteristic pattern of subcellular distribution. Each of the variants displays a dramatic reduction in DNA-binding activity, as measured using transiently transfected Cos7 cell lysate and a consensus estrogen response element (ERE) in an *in vitro* gel mobility shift assay. While this DNA-binding defect appears to be complete for ERΔE2, ERΔE3, ERΔE4, and ERΔE6, weak DNA-binding is observed for ERΔE5 and ERΔE7, but only when DNA complex formation is stabilized by the addition of a bivalent antibody capable of recognizing the N-terminus of the ERα protein. Scatchard analysis of hormone-binding demonstrates that ERΔE3 binds 17β-estradiol (E_2) with a similar affinity to wild type ERα (wt ERα). Individual variants co-transfected with the pERE-TK-

CAT reporter gene (a consensus ERE-driven CAT reporter gene that is highly responsive to E_2-liganded wt ERα) were ineffective at inducing CAT expression in ER-negative HeLa cells. Only ERΔE5 showed indications of positive transcriptional activity on the pERE-TK-CAT reporter, but always this activity was limited to approximately 5% of the activity of wt ERα. When variants were expressed simultaneously with wt ERα, ERΔE3 and ERΔE5 were observed to have a dominant negative effect on wt ERα transcriptional activity. More compelling evidence that ERα splicing variants are transcriptionally active is observed with transfection experiments using a non-consensus estrogen-regulated reporter gene construct. Transcription from a region of the ovalbumin promoter, which contains an ERE half site and an AP-1 motif, is positively regulated by liganded wt ERα and ERΔE3 in phorbol ester-treated, transiently transfected HeLa cells.

INTRODUCTION

Estrogens are crucial for normal physiological development of the breast and this class of hormones plays a central role in breast oncology (1-5). Binding of estrogen to the estrogen receptor (ER) elicits a change in receptor conformation that allows the receptor to bind DNA and enhance transcription from the promoters of regulated genes (6-8). ER-induced gene expression supports the proliferation and ultimately, the differentiation of target cells (2,3). Interference with these proliferative effects forms the basis for the chemotherapeutic actions of estrogen antagonists that are used to treat cancers of the breast and reproductive tract (4). The reported success of anti-estrogens such as tamoxifen and raloxifene in preventing breast tumors emphasizes a crucial role for ER in mammary carcinogenesis (1,5,9).

The transcriptional effects of estrogens are mediated by two closely related receptor isoforms, ERα and the more recently described ERβ (10,11), each of which is encoded by a separate gene. While ERβ is also being investigated for its potential role in various diseases including cancer, this study focuses solely on the ERα isoform. Analysis of mRNA prepared from a variety of estrogen responsive cells and tissues, including breast tumors, has established that ERα mRNA is typically expressed as a mixture of transcripts (12-15). This heterogeneity results largely from a pattern of alternative mRNA splicing that gives rise to a family of correctly processed and exon-skipped ERα mRNAs. ERα mRNA comprises sequences from 8 coding exons and is translated to yield

a protein with discrete functional domains. An N-terminal transactivation function (AF1) encoded by exon 1 and a portion of exon 2 is thought to promote gene transcription by interacting with nuclear receptor coactivators and also with proteins integral to the transcription initiation complex (6,16,17). Derived from exons 2 and 3 is a centrally located zinc-finger motif (commonly referred to as the DNA-binding domain or DBD) that is essential for sequence-specific DNA-binding and transcriptional activation through canonical estrogen response elements (EREs) (18). Within the region encoded by exon 4 are the nuclear localization signals (NLS) and a hinge region which allows for receptor conformational flexibility (8,19). A ligand-binding domain (LBD) confers regulatory function to the receptor and is encoded by the C-terminal exons 4 through 8 (20). Exon 8 also includes determinants for subunit dimerization and a well characterized C-terminal transactivation function (AF2) which promotes gene transcription by recruiting coactivators (6,7,8,21). Like other nuclear receptors, ERα is a modular protein in that individual domains are capable of demonstrating autonomous function within receptor mutants, as well as when they are introduced into heterologous fusion proteins (6,18). It can reasonably be assumed that the exclusion of a particular exon will predictably result in a protein lacking the function ascribed to that exon. Additionally, it is probable that the loss of a particular exon will result in unpredictable functional deficits or perhaps even bestow a novel function on the variant receptor. This study examines the function of ERα splicing variants from the vantage point of what is known about the functional organization of wt ERα. We report results from

experiments designed to assess receptor capacity to translocate to the nucleus, bind to DNA, bind ligand and promote gene transcription.

Fuqua and colleagues have reported that ERΔE5 (which contains the AF1 domain, but lacks AF2 and the regulatory functions imparted by the LBD), is constitutively active in promoting transcription from an ERE in a heterologous yeast reporter gene assay (22,23). These authors have also described that over-expression of ERΔE5 in a stably transfected breast cancer cell line (MCF-7) supported greater proliferation compared to control cells, as well as imparting a tamoxifen resistant phenotype (24). In the human osteosarcoma cell line U2-OS, it has recently been reported that co-expression of ERΔE5 significantly enhances ERE-directed reporter gene expression induced by wt ERα (25). The existence of a constitutively active receptor variant (such as ERΔE5) able to exert a mitogenic effect in breast tumor cells in the absence of E_2 or in the presence of tamoxifen is an appealing explanation for the acquisition of anti-estrogen resistance observed in previously responsive tumors and cell lines (26,27). However, this model is challenged by conflicting observations that ERΔE5 and closely related, genetically engineered ERα mutants do not efficiently induce transcription from an ERE reporter in transiently transfected ER-negative HeLa or CEF cells (7,28), or promote proliferation in stably transfected MCF-7 cells (28).

Recently a novel mechanism for mediation of an estrogen response has been reported to involve AP-1 directed regulation of transcription by ER (29-32).

AP-1 describes the fos / jun family of transcription factors that play a key role in transducing the effects of growth factors to regulate cell proliferation (33,34). A variety of estrogen-responsive genes have been described that lack a palindromic ERE, but instead contain one or more consensus AP-1 elements (5'-TGAG/CTCA-3'), with or without a degenerate ERE or ERE half site (5'-GGTCA-3' or 5'-TGACC-3'). Examples of such genes include ovalbumin which is induced by E_2 in chicken oviduct cells (35) and the insulin-like growth factor-I (IGF-I) gene whose expression is stimulated by E_2 treatment in cultured rat osteoblasts (36). An AP-1 enhancer motif identified in the chicken IGF-I promoter is essential for E_2 and phorbol ester stimulated gene transcription (31). Phorbol esters act directly on protein kinase C to initiate a signal transduction cascade that ultimately activates AP-1 (33). Reporter gene co-transfection studies with expression vectors for AP-1 isoforms and ERα in HeLa cells indicate that a similar mechanism regulates the human collagenase promoter (32). The minimal region of the collagenase promoter reported to be responsive to tamoxifen-liganded wt ERα, and to a variety of ERα mutants harbors a critical AP-1 element and lacks a consensus ERE. Additionally, the activity of ERα on the collagenase promoter was enhanced with AP-1 (c-jun or c-fos) over-expression (32). Further evidence that ER regulation converges with AP-1 directed gene transcription is provided by results from protein binding assays indicating that c-jun is able to bind to wt ERα *in vitro* (32).

Although evidence for function of ERα variants has been elusive, reports that ERΔE5 can support weak, cell type-dependent activity (23,25,28), and that, when tested on an ERE, both ERΔE5 and ERΔE3 are dominant negative receptor forms in the presence of wt ERα (37,38) indicate that it is inaccurate to label these variants as transcriptionally inert. To investigate the capacity for ERα splicing variants to regulate gene transcription we have expanded our transcriptional focus to include the noncanonical ovalbumin promoter in addition to the consensus ERE. Here we present data indicating that individual variants display both similarities and differences compared with wt ERα, and that selected splicing variants (specifically ERΔE3 and ERΔE5) have the capacity to both positively and negatively regulate gene expression, depending on the cell and promoter context.

RESULTS

Construction of plasmids for efficient expression of individual ERα mRNA splicing variants

Numerous variant ERα cDNAs have now been cloned and sequenced from breast tumors and established tumor cell lines (12-15). The most common variants harbor a precise deletion of one of the internal exons from the eight that contribute to the structure of the mature ERα protein, suggesting that they arise as a result of imprecise splicing of the primary ERα mRNA transcript. ERα cDNAs with sequence deletions corresponding to exons 2, 3, 4, 5 and 7 have been identified, along with a large number of more complex variants (12-15). These basic variants will be referred to as ERΔE2 through ERΔE7, where the deleted exon is indicated numerically. While there has been extensive analysis at the RNA level of the pattern of expression and abundance of ERα splicing variants, limited information is available on their functional activity. We have therefore constructed CMV promoter driven ERα cDNA expression vectors representing the exon-skipped variants ERΔE2 through ERΔE7 to enable their functional characterization in a well defined cell transfection system. Our assembled pool of ERα splicing variants also includes the hypothetical receptor ERΔE6 which is not readily identified *in vivo*. Figure 1 diagrams the ERα mRNA splicing variants showing the positions of deleted exons and their consequences with respect to protein structure. Deletion of exon 2, 5, 6 or 7 all

cause a frame-shift mutation resulting in premature termination of translation, thereby generating a diverse class of C-terminally truncated receptor forms. Omission of either exon 3 or 4 does not disrupt the mRNA reading frame, but produces a receptor protein with an internal deletion.

Transient expression in Cos7 cells demonstrates that each of these variants translates to a stable protein able to accumulate to readily detectable levels within transfected cells (Fig. 2). Based on immunoblot analysis with an N-terminal monoclonal antibody (Mab-17), which recognizes an epitope common to each of the variants (39), we observe the mobility of the six variant proteins is consistent with its predicted molecular weight.

Measurement of the DNA-binding activity of the ERα splicing variants

Efficient DNA binding by ERα requires the cooperation of several functional elements within this protein, including the centrally located DNA-binding domain and a ligand-inducible subunit dimerization motif located near the C-terminal end of the LBD (7,18). It is also possible that additional subunit contacts occur elsewhere in the protein. Because all of the ERα splicing variants sustained deletions within various regions of this protein, it was of interest to systematically assess the DNA-binding ability of each variant. For this purpose, gel mobility shift assays were performed using extracts prepared from E_2 treated, transiently transfected Cos7 cells. Extracts were incubated with a ^{32}P-labeled oligonucleotide containing a consensus estrogen response

element (AGGTCACAGTGACCT) from the xenopus vitellogenin A2 promoter. As expected, variants that harbor a mutation within the DBD (ERΔE2 and ERΔE3) are completely unable to recognize the ERE (Fig. 3, lanes 5-8). Less predictably, the loss of exons contributing to the LBD also result in a strong defect in ERE recognition (Fig. 3, lanes 9-16). For ERΔE5 and ERΔE7, however, this appears to be a quantitative defect in DNA binding. The addition of the monoclonal antibody, MAb-17, to the binding reactions consistently results in the recovery of weak DNA binding by ERΔE5 (Fig. 3, lane 12). Presumably the role of the bivalent antibody is to stabilize the interaction of receptor subunits with their palindromic binding site, mimicking the function of the missing dimerization motif present within the C-terminus of the LBD. These results suggest the possible existence of cell-specific constituents that perform the same function *in vivo* and may account for the variable activity of ERΔE5 and related constructs in different cell types (7,25,28). We have also observed the formation of a complex between ERΔE7 and labeled ERE probe (Fig. 3, lane 16). The relative weakness of DNA-binding observed in these studies raises serious questions about the extent to which ERΔE5 and ERΔE7 are able to recognize and bind to a consensus ERE, *in vivo*.

ERΔE3, like wt ERα binds ligand

To test the ability of the ERα mRNA splicing variants to bind hormone we performed a saturation binding assay on whole cell extracts from Cos7 cells transiently transfected with wt ERα or the ERα variants. Only wt ERα and ERΔE3 were able to bind ^{3}H-labeled E_2 whereas all of the remaining variants demonstrated no specific ligand binding (Fig. 4A). The individual deletion of exons 2, 4, 5, 6 and 7 effectively eliminates all, or a significant portion of the LBD, consistent with their loss of hormone binding. We confirmed these results using an *in vivo* ligand-binding assay where the binding of a fluorescent estrogen analogue was visualized in cells cultured on cover slips. Cos7 cells transiently expressing the individual variants or wt receptor were treated with the fluorescent ligand, nitrile tetrahydrochrysene (nitrile THC) (40). Only those cultures transiently transfected with wt ERα or ERΔE3 were observed to stain with this ligand. In both cases, staining was localized tightly within the nucleus. This suggests that among the variants examined, wt ERα and ERΔE3 exclusively bind ligand and in both cases, ligand-bound receptors are translocated normally to the nucleus of expressing cells (Fig. 4B). With Scatchard analysis we compared the affinity of ERΔE3 and wt ERα for ^{3}H-labeled E_2. The measured dissociation constants were 0.66 nM for wt ERα and 0.79 nM for ERΔE3 (Fig. 4C).

Subcellular localization of ERα splicing variants

To more carefully assess the subcellular localization of ERα splicing variants, including those that fail to bind ligand, Cos7 cells were transiently transfected with expression vectors encoding wt ERα or individual variants. These receptors were detected in transfected cells by indirect immunofluorescence staining (using the MAb-17 monoclonal antibody) and confocal microscopy. Similar to wt ERα, ERΔE3 and ERΔE5 localize to the nuclei of transfected cells, though ERΔE5 showed perinuclear as well as nuclear staining (Fig. 5). These results are consistent with the fact that each of these receptors retains the NLS immediately downstream of the DBD (8,19). Subcellular localization studies have also been completed for the exon 2,4,6 and 7 deletion variants. Each of these proteins can be readily detected in transfected cells, but they all possess dramatic defects in nuclear targeting (Fig. 5). Nuclear targeting of wt ERα is governed in large part by a tripartite karyophilic signal present within exon 4 (19). Loss of this signal is therefore consistent with the cytoplasmic pattern of distribution of mutants such as ERΔE2 and ERΔE4, both of which lack protein corresponding to exon 4 sequences. Inappropriate presentation or folding of this signal must account for the defects in nuclear localization seen with ERΔE6 and ERΔE7, since the NLS is retained in these variants.

Characterization of the Transactivation Function of ERα splicing variants on an ERE

HeLa cell co-transfection experiments designed to assess the transcriptional activity of individually expressed ERα splicing variants have failed to demonstrate any significant ability of variant receptors to support gene activation through an ERE, with the possible exception of the ERΔE5 variant which appears to display a low level of constitutive transcriptional activity on an ERE driven reporter in some, but not all cell types examined (23,25,28). In our hands none of the variants were effective transcriptional activators of an ERE containing promoter. ERΔE5 repeatedly showed only modest constitutive activity (approximately 5% of wt ERα induction) on an ERE-directed reporter plasmid co-transfected into HeLa cells (see Fig. 7, insert).

It is important to acknowledge that the tissues and cell lines that express these variants also express wt ERα. We have previously reported that the ERΔE3 variant acts as a dominant negative mutant when it is co-expressed with wt ERα in HeLa cells treated with E_2 (38). In the human breast epithelial cell line HMT-3522S1, ERΔE5 has also been reported to disrupt transactivation by agonist-bound wt ERα of an ERE reporter gene (37). To clarify whether this is a function unique to these variants we completed a series of experiments to assess the likelihood that the remaining exon-skipped ERα variants also support transcriptional inhibitory effects. When tested in a HeLa cell co-

transfection assay in which the expression of pERE-TK-CAT was driven by E_2-bound wt ERα, a 5-fold molar excess of any of the splice variants lacking exons 2,4,6, or 7 failed to inhibit the E_2-dependent induction of CAT gene expression by intact receptor (data not shown). In agreement with previously published results, the ERΔE3 and ERΔE5 variants both demonstrated a dominant inhibitory activity at all molar ratios tested (Fig. 6). With the caveat that equal amounts of plasmid DNA support similar levels of variant receptor expression (see Fig. 2), it appears that ERΔE3 and ERΔE5 are nearly equivalent in their inhibitory activity in HeLa cells.

ERΔE3 variant is a positive regulator of gene expression

The results presented above indicate that ERα splicing variants are either inactive (ERΔE2, ERΔE4, ERΔE6 and ERΔE7) or largely inhibitory (ERΔE3 and ERΔE5) in their effect on reporter constructs that contain a consensus ERE. Recent reports suggest that wt ERα is able to transactivate genes whose promoters do not contain an obvious ERE. In particular are promoters such as those that drive expression of the collagenase and ovalbumin genes that are regulated by wt ERα and contain a critical AP-1 element (29,32). Mutational analysis revealed that the DBD was not required for ERα-dependent expression of these genes. Clearly the mechanisms of ERα transcriptional activity and DNA targeting are complicated by these reports. We

propose that in order to assess the transactivating potential of ERα variants, the promoter focus must be expanded to include those containing non-canonical regulatory elements. These thoughts prompted us to test the activity of the ERα variants on the ovalbumin promoter which contains a complex hormone response element. We performed co-transfection experiments in HeLa cells using vectors expressing wt ERα or the exon-skipped variants ERΔE2 through ERΔE7 and a reporter construct driven by a fragment of the ovalbumin promoter (-1342 to +7 relative to transcription start site) described to encompass much of the regulatory sequence of this gene (35,41). Results from these experiments indicate that both wt ERα and ERΔE3 support inducible gene expression from the ovalbumin promoter (Fig. 7) and that all of the remaining single exon-skipped variants are transcriptionally inactive on this reporter construct (data not shown). For wt ERα, this is consistent with previously published reports (29,35). Maximal activity was measured in cultures treated with both phorbol 12-myristate, 13-acetate (PMA, a phorbol ester) and E_2, where a 16-fold induction was observed. Similar to wt ERα, ERΔE3 supported a marked 9-fold induction of this reporter, despite its lack of an intact DBD. In both cases the result of co-treatment with PMA and E_2 is highly synergistic as E_2 treatment alone has no significant effect and PMA treatment alone supports only modest induction for wt ERα (2.5-fold relative to vehicle control). Tamoxifen treatment of wt ERα- or ERΔE3-transfected cultures, either alone or together with phorbol ester, had no

significant effect on pOvalb-CAT expression. In those cells not expressing ERα protein (i.e., controls transfected with an empty CMV expression vector), there was no PMA induction. This suggests that, in the absence of ERα, activated AP-1 alone does not support transcription from the ovalbumin promoter in these cells. Regulation of the reporter is only observed with PMA treatment when an ERα isoform is co-expressed.

DISCUSSION

Our efforts to functionally characterize exon-skipped ERα mRNA splicing variants have identified two receptor isoforms that possess the ability to modulate estrogen signaling on genes that are targeted by the estrogen receptor. Although their protein structure is significantly altered, the ERΔE3 and ERΔE5 splicing variants retain many of the activities attributed to the full length receptor. Loss of exon 3 results in a receptor protein with an internal deletion that lacks a major portion of the DBD and therefore prevents ERΔE3 from binding to a consensus ERE, as confirmed by gel mobility shift analysis. However, ERΔE3 retains the LBD and NLS thereby allowing it to bind hormone with an affinity similar to wt ERα and translocate to the nucleus. The deletion of exon 5 causes a frame-shift mutation and results in a C-terminally truncated form of the receptor. Loss of the LBD predictably renders ERΔE5 unable to bind E_2. Nonetheless, ERΔE5 also retains the NLS and immunofluorescence analysis shows nuclear staining in Cos7 cells transfected with this variant.

Rather than serving to stimulate transcription on a consensus ERE, results from transient transfection experiments in HeLa cells that combine either ERΔE3 or ERΔE5 with wt ERα and an ERE-driven reporter gene indicate that these isoforms actually function to inhibit transcriptional activation by wt ERα. These observations agree with our previous results and with those reported by others from similar experiments using HMT-3522S1 cells (37,38). A 70%

inhibition of transcriptional activation by E_2-liganded wt ERα on an ERE-driven CAT reporter gene was observed in HeLa cells when ERΔE3 and wt ERα expression vectors were co-transfected at a ratio of 5 to 1 (38). In the ER-negative cell line HMT-3522S1, co-expression of an equal amount of ERΔE5 significantly inhibited stimulation of an ERE reporter construct by wt ERα (37). Increasing the ratio of transfected variant to wt ERα demonstrates that the repression of wt ERα by ERΔE3 and ERΔE5 is dose-related and becomes nearly complete when the variants are present in sufficient excess (37,38), a situation that is reported to occur in some breast tumor cells (22). The dominant negative character of ERΔE3 and ERΔE5 suggests that, like wt ERα, these variants are able to interact with at least one component of the ERE-directed transcription complex in a manner that disrupts positive gene regulation by wt ERα. Based on gel mobility shift assay analysis, it is unlikely that transcriptional interference by these variants involves binding to an ERE to the exclusion of wt ERα. Our DNA-binding analysis indicates that ERΔE5 can bind only weakly to DNA, and only when the formation of this complex is stabilized by the addition of a bivalent antibody. The role of the antibody in this case is presumably to substitute for the missing dimerization interface and to tether receptor subunits together in a form more able to interact with DNA. DNA binding by ERΔE7 similarly requires the addition of antibody, but this binding is even less efficient than binding by ERΔE5. Interestingly, a correlation exists among the ERα

variants between their failure to translocate to the nucleus and their inability to inhibit wt ERα activity in mammalian cells (data not shown). As of yet, no clear function has been established for the ERΔE7 variant in mammalian cells despite an earlier report that ERΔE7 is a dominant inhibitor of wt ERα function in yeast (42). This is noteworthy since a number of quantitative studies have indicated that, as a rule, ERΔE7 represents the most abundant of the ERα splicing variants (summarized in 15).

We have previously reported that although ERΔE3 is unable to bind to an ERE itself, it can prevent wt ERα from binding to DNA (38). That ERΔE3 inhibits both DNA complex formation and transactivation by wt ERα suggests that the potential targets of interaction by ERΔE3 may include protein-protein contacts with wt ERα itself or interactions with nuclear receptor coactivators or other receptor-associated factors. ERα function may be disrupted when ERΔE3, which lacks the DBD but retains the hormone-inducible dimerization domain, forms mixed dimers with wt ERα that are inefficient at binding stably to DNA. We are able to show that, in the presence of E_2, ERΔE3 can form a stable complex with the ligand-binding domain of ERα fused to glutathione-S-transferase (GST), consistent with this model for inhibition by ERΔE3 (Bollig and Miksicek, unpublished data). We also observe that *in vitro* translated ERΔE3 is able to associate in an E_2-dependent manner with a fragment of steroid receptor

coactivator-1 (SRC-1) containing the nuclear receptor (NR) boxes (amino acids 570-780) (43). SRC-1 is a potent enhancer of E_2-bound ERα and is required for its full transcriptional activity (44,45). A site for SRC-1 interaction within wt ERα corresponds with the AF2 domain (46), a region that is retained in the ERΔE3 variant. Transfection experiments in E_2-treated HeLa cell cultures demonstrate that co-expression of mutants containing the C-terminus of ERα can attenuate ERα-dependent gene expression and that this decreased activity can be overcome with simultaneous over-expression of the SRC-1-related coactivator TIF2 (47). These results suggest that coactivators are limiting factors for which the receptors are competing and that ERΔE3, like wt ERα, is a target for SRC-1 binding.

In a surprising result from co-transfection studies using engineered mutants of ERα, maximal expression of an ERE-containing reporter gene could be observed when SRC-1 was transfected simultaneously with separate N- and C-terminal fragments of ERα, containing the AF-1/DBD and the LBD/AF2 regions, respectively (48). This indicates that separate AF1- and AF2-containing ERα polypeptides can interact in a transcriptionally productive manner, provided they are brought together by SRC-1. These results offer initial indications that SRC-1 interacts separately and perhaps directly with both the AF1 and AF2 domains. More support for this is provided by recent reports that SRC-1 binds N-terminal polypeptides from ERα in solution (17). This

suggests the possibility that the inhibitory function of ERΔE5, which itself is relatively inefficient at binding DNA or activating transcription through an ERE, may result from competition with wt ERα for interaction with SRC-1 or other cellular factors.

The most compelling evidence that several of the ERα mRNA splicing variants may indeed be transcriptionally active is seen in transfection experiments involving ERΔE3 and reporter gene constructs containing a non-consensus hormone regulatory element. Recently a novel mechanism for mediation of an estrogen response has been reported to involve AP-1 directed regulation of transcription by ERα (29-32). AP-1 and its isoforms represent a family of nearly ubiquitous transcription factors whose activity is crucial for the efficient expression of a wide variety of genes. As an important downstream target for the MAPK signaling cascades, AP-1 is a central player in mediating the effects of serum and growth factors on cellular proliferation (33,34). A variety of estrogen-responsive genes have been described that lack a palindromic ERE, but instead contain one or more consensus AP-1 elements (5'-TGAG/CTCA-3'), with or without a nearby degenerate ERE or ERE half site (5'-GGTCA-3' or 5'-TGACC-3') (29,31,32). It should be noted that these imperfect EREs may in some cases serve dual function as cryptic AP-1 response elements whose consensus sequence bears superficial similarity to an ERE half-site (29,31,32).

An important observation from the analysis of genes regulated by non-canonical EREs is that the structure-activity requirements for activation by ERα (both for the ligand and for the receptor) are different than those for transcriptional activation through a palindromic ERE. Using a region of the collagenase gene promoter (-73/+63) that lacks an ERE but harbors an essential AP-1 element, Kushner and coworkers demonstrated that a DBD-deleted mutant of ERα was significantly more effective at supporting E_2-induced reporter gene expression than wt ERα in transfected HeLa cells (32). Similar to collagenase gene expression, ERα-dependent activation of the chicken ovalbumin promoter, which lacks a palindromic ERE, does not require an intact DBD (29). Furthermore, an ERE half-site was determined to be the site for synergistic regulation of the ovalbumin gene expression by AP-1 and ERα (29). Our studies involving co-transfection of an ERα splice variant with the ovalbumin promoter construct, pOvalb-CAT, agree with these findings. Results from Fig.7 demonstrate that, compared with mock-transfected HeLa control cultures, pOvalb-CAT is strongly activated by either wt ERα or ERΔE3. A breakdown of treatments indicates that maximal activity of both wt ERα and ERΔE3 clearly requires E_2 in addition to an AP-1 activator. Tamoxifen treatment had little or no effect on their activity, either with or without PMA co-treatment. This contrasts with results observed when wt ERα was co-transfected with a collagenase reporter construct in HeLa cells, where tamoxifen supported a

significant induction of reporter gene expression (32). The differences we observe between the relative activities of ERα isoforms on the ovalbumin promoter and the activation requirements reported for other AP-1 directed ERα regulated genes highlight the complexity of ERα signaling through this mechanism.

Our transfection results show that, while several of the ERα splicing variants are functionally incapacitated by their deletions, two of the variants clearly retain significant transcriptional activity. For the ERΔE3 and ERΔE5 variants, this activity is quite complex. Both of these variants represent stable receptor isoforms that, like the full-length receptor, localize efficiently to the nucleus where they can interact with the transcription apparatus. However, when acting through a consensus ERE, these variants completely lack (ERΔE3) or show only weak (ERΔE5) transcriptional stimulatory activity, consistent with their poor DNA-binding ability. On the contrary, both variants serve to blunt the ability of co-expressed wt ERα to promote transcription of ERE-containing genes. At the same time, the ability of ERΔE3 (and presumably also ERΔE5) to interact productively with nuclear receptor co-activators or other transcription factors gives these ERα splicing variants the potential to stimulate or otherwise modulate gene expression through non-consensus hormone response elements that are targeted by AP-1 motifs or other DNA-binding sites. We have clearly shown this to be true for ERΔE3 and the chicken ovalbumin promoter

and believe that this is also likely to be true for many other genes, such as those encoding collagenase, cathepsin D, IGF-1, TGF-β, c-fos, HSP-27, and RAR-α, that lack an obvious ERE and yet still respond to estrogen. In this respect, ERα splicing variants may actually serve to redirect transcription away from ERE-containing genes to genes such as these that appear to be regulated non-classically by estrogens.

MATERIALS AND METHODS

Expression Vectors

Plasmids for ERα mRNA splicing variant cDNAs were generated as derivatives of pCMV4 (49) and pcDNA3.1 (InVitrogen Corp., San Diego, CA), which support high levels of receptor expression in HeLa and Cos7 cell lines (40). Plasmids expressing ERΔE4, ERΔE5, and ERΔE6 were generated using synthetic oligonucleotides to construct the variant splice junctions within an otherwise wt ERα cDNA expression plasmid. The remaining plasmids were constructed with the use of flanking restriction sites to shuttle cloned cDNAs (38) into the appropriate expression vectors.

Cell Culture, Transfection and CAT Assay

Cos7 and Hela cells were grown in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf-serum, 5 mM HEPES (pH 7.4), 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml). Cells were transfected by the $CaPO_4$ method, as previously described (50). HeLa cells (approximately 2 x 10^{-6} cells per 100 mm dish) were transfected with 1 µg of the indicated ERα isoform expression plasmid and 16 µg of the estrogen responsive reporter plasmid, pERE-TK-CAT (51) or pOvalb-CAT (a reporter gene construct containing -1342 to +7 bp of the chicken ovalbumin promoter relative to transcription start site) (41). Calf thymus DNA (10 µg) was added as carrier. Following over-night incubation with DNA, culture medium was

replaced with 5% charcoal-treated serum supplemented DMEM containing the indicated hormones. After 24 hours incubation, cells were harvested and CAT assays were performed as previously described (52) using 100 μg protein. Quantification was performed by phosphorimage analysis of thin layer chromatographs (ImageQuaNT, Molecular Dynamics, Sunnyvale, CA). Cos7 cells were similarly transfected with 10 μg of the indicated expression plasmid and 10 μg of calf thymus carrier DNA. After over-night culture with DNA, cells were cultured for 48 hours in 10% calf serum supplemented DMEM.

Estradiol Binding Analysis

Ligand-binding assays were performed as previously described (39). Whole cell extracts were prepared from transfected Cos7 cells that were resuspended and sonicated in extraction buffer. Aliquots containing 200 μg of protein were incubated over-night at 4°C with various concentrations (0.1 nM - 10 nM) of ^{3}H-labeled 17β-estradiol (New England Nuclear) in the presence or absence of a 200-fold molar excess of unlabeled 17β-estradiol. Free ligand was separated from bound ligand by treatment with dextran-coated charcoal. For determination of equilibrium binding constants, these data were plotted according to the method of Scatchard (53).

DNA-Binding Assays

DNA-binding assays were performed as previously described (39). Aliquots containing 30 μg of protein from extracts prepared as above from transfected Cos7 cells were pre-incubated for 15 min at room terperature in 10 μl binding buffer [10 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 1 mM dithiothreitol, and 20% glycerol] containing 1 μg poly (dI-dC), with or without added hER-specific monoclonal antibody (Mab17), generated as described by Neff (39). Approximately 6 fmol (40,000 cpm) of a ^{32}P-labeled double stranded ERE oligonucleotide (38) were added to the samples and incubated for 30 min at room temperature, followed by an additional 5 min incubation at 4°C. Samples were then loaded on a pre-electrophoresed non-denaturing 5% polyacrylamide gel that was run in 0.5 x Tris-Borate-EDTA at 275 V for 2 h. The gel was dried and exposed for autoradiography.

Immunoblot Analysis

Discontinuous 12% SDS-PAGE was carried out as previously described (54). After electrophoresis of 30 μg of protein from transfected Cos7 cell whole cell extracts, proteins were electrophoreticallly transferred to nitrocellulose filters with a Bio-Rad Trans Blot apparatus (Bio-Rad Laboratories, Richmond, CA) using the procedure of Erickson *et al.* (55). Immunoblots were probed with the ER-specific monolclonal antibody, Mab17 (39). Immunoreactive protein was visualized using enhanced chemiluminescence, following manufacturer's instructions (Amersham, Arlington Heights, IL).

Immunohistochemical and Cytochemical Analysis

Indirect immunofluorescence analysis was performed on Cos7 cells that were plated and transfected on glass cover slips. On the second day following transfection, cells were washed three times with Tris-buffered saline (TBS), fixed for 3 min in cold 95% methanol, rehydrated by three washes with TBS, and incubated 30 min at 37°C with primary antibody (Mab17 hybridoma supernate used at a 1:10 dilution in TBS). Bound antibody was detected by staining with a rhodamine-conjugated affinity-purified goat anti-mouse immunoglobulin G (Boehringer Mannhein, Indianapolis, IN) diluted 1:2000 in TBS, incubating for 30 min at 37°C in the presence of 0.02 μg/ml of 4′, 6-diamidine-2-phenylindole dihydrochloride. Confocal images were recorded using the Odyssey system (Noran Instruments, Middleton, WI) on an Optiphot 2 Nikon microscope. Fluorescent ligand staining of transfected Cos7 cells was performed as described by Miksicek et al. (40), on live, whole-cell mounts treated in DMEM with 10^{-7} M nitrile THC. For these studies cells were visualized using a Nikon UFX microscope equipped with a 100 watt mercury lamp for fluorescence excitation, and a 40x 0.7 numerical aperture Plan objective.

Acknowledgements:

We are grateful to M. Sanders for kindly providing pOvalb-CAT. We would also like to thank M. Morrison and D. Ankrapp for helpful suggestions during the course of this work. This work was supported by award DAMD1794-J-4372 to RJM from the US Army Breast Cancer Research Program .

REFERENCES

1. Clarke M, Collins R et al. 1998 Tamoxifen for early breast cancer: an overview of randomised trials. Lancet 351:1451-1467

2. Dickson Rb, Johnson MD, el-Ashry D, Shi YE, Ban O, Zugmaier G, Ziff B, Lippman ME, Chysogelos S 1993 Breast Cancer: Influence of endocrine hormones, growth factors and genetic alterations. Adv Exp Med Biol 330:119-141

3. Haslam SZ. 1987 Role of sex steroid hormones in normal mammary gland function. In: Neville MC and Daniel CW (eds) The Mammary Gland. Plenum, New York, pp 499-533

4. Kaufmann M 1997 A review of endocrine options for the treatment of advanced breast cancer. Oncology 54 Suppl 2:2-5

5. Smigel K 1998 Breast cancer prevention trial shows major benefit, some risk. J Natl Cancer Inst 90:647-648

6. Beato M, Sanchez-Pacheco A 1996 Interaction of steroid hormone receptors with the transcription initiation complex. Endocr Rev 17:587-609

7. Kumar V, Chambon P 1988 The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145-156

8. Tsai M-J, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451-486

9. Huston L 1999 Raloxifene reduces breast cancer risk. Lancet 353:44

10. Kuipper GGJM, Enmark E, PeltoHuikko M, Nilsson S, Gustafsson JA 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci (USA) 93:5925-5930

11. Mosselman S, Polman J, Dijkema R 1996 ERβ: Identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49-53

12. Castles CG, Klotz DM, Fuqua SAW, Hill SM 1995 Coexpression of wild-type and variant oestrogen receptor mRNAs in a panel of human breast cancer cell lines. Br J Cancer 71:974-980

13. Gotteland M, Desauty G, Delarue JC, Liu L and May E 1995 Human estrogen receptor messenger RNA variants in both normal and tumor breast tissues. Molecular and Cellular Endocrinology 112:1-13

14. Miksicek RJ, Lei Y, Wang Y 1993 Exon skipping gives rise to alternatively spliced forms of the estrogen receptor in breast tumor cells. Breast Cancer Res Treat 26:163-179

15. Murphy LC, Leygue E, Dotzlaw H, Douglas D, Coutts A, Watson PH 1997 Oestrogen receptor variants and mutations in human breast cancer. Ann Med 29:221-234

16. Metzger D, Ali S, Bonnert JM, Chambon P 1995 Characterization of the amino-terminal transcriptional activation function of the human estrogen receptor in animal and yeast cells. J Biol Chem 270:9535-9542

17. Webb P, Nguyen P, Shinsako J, Anderson C, Feng W, Nguyen MP, Chen D, Katzenellenbogen BS, Stallcup MR, Kushner PJ 1998 Estrogen receptor activation function 1 works by binding p160 coactivator proteins Mol Endocrinology 12:1605-1618

18. Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P 1987 Functional domains of the human estrogen receptor. Cell 51:941-951

19. Ylikomi T, Bocquel MT, Berry M, Chambon P 1992 Cooperation of protosignals for nuclear accumulation of estrogen and progesterone receptors. EMBO J 11:3681-3694

20. Kumar V, Guren S, Staub A, Chambon P 1986 Localization of the oestradiol-binding and putative DNA binding domain of the human oestrogen receptor. EMBO J 5:2231-2236

21. Horowitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L 1996 Nuclear receptor coactivators and corepressors. Mol Endocrinol 10:1167-1177

22. Castles CG, Fuqua SAW, Klotz DM, Hill SM 1993 Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line. Cancer Research 53:5934-5939

23. Fuqua SAW, Fitzgerald SD, Chamness GC, Tandon AK, McDonnell DP, Nawaz Z, O'Mally BW and McGuire WL 1991 Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Research 51:105-109

24. Fuqua SAW and Wolf DM 1995 Molecular aspects of estrogen receptor variants in breast cancer. Breast Cancer Res Treat 35:233-241

25. Chaidarun SS, Alexander JM 1998 A tumor-specific truncated estrogen receptor splice variant enhances estrogen-stimulated gene expression. Mol Endocrinol 12:1355-1366

26. Wiseman LR, Johnson MD, Wakeling AE, Lykkesfeldt AE, May FEB, Westley BR 1993 Type I IGF receptor and acquired tamoxifen resistance in oestrogen-responsive human breast cancer cells. Eur J Cancer 29A:2256-2264

27. Wolf DM and Jordan VC 1994 Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice. Breast Cancer Res Treat 31:117-127

28. Rea D and Parker MG 1996 Effects of an exon 5 variant of the estrogen receptor in MCF-7 breast cancer cells. Cancer Research 56:1556-1563

29. Gaub MP, Bellard M, Scheuer I, Chambon P and Sassone-Corsi P 1990 Activation of the ovalbumin gene by the estrogen receptor involves the Fos-Jun complex. Cell 63:1267-1276

30. Uht RM, Anderson CM, Webb P and Kushner PJ 1997 Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element. Endocrinology 138:2900-2908

31. Umayahara Y, Kawamori R, Watada H, Imano E, Iwama N, Morishima T, Yamasaki Y, Kajimoto Y and Kamada T 1994 Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J Biol Chem 269:16433-16442

32. Webb P, Lopez GN, Uht RM and Kushner PJ 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol 9:443-456

33. Boyle WJ, Smeal T, Defize LHK, Angel P, Woodgett JR, Karin M and Hunter T 1991 Activation of protein kinase C decreases phosphorylation of cJun at sites that negatively regulate its DNA-binding activity. Cell 64:573-584

34. Westwick JK, Weitzel C, Minden A, Karin M and Brenner DA 1994 Tumor necrosis factor a simulates AP-1 activity through prolonged activation of the c-Jun kinase. J Biol Chem 269:2639-26401

35. Tora L, Gaub MP, Mader S, Dierich A, Bellard M, Chambon P 1988 Cell-specific activity of a GGTCA half-palindromic oestrogen-responsive element in the chicken ovalbumin gene promoter. EMBO J 7:3771-3778

36. Ernst M, Rodan GA 1991 Estradiol regulation of insulin-like growth factor-1 expression in osteoblastic cells: evidence for transcriptional control. Molecular Endocrinology 5:1081-1089.

37. Ohlsson H, Lykkesfeldt AE, Madsen MW, Briand P 1998 The estrogen receptor variant lacking exon 5 has dominant negative activity in the human breast epithelial cell line HMT-3522S1. Cancer Research 58:4264-4268

38. Wang Y and Miksicek RJ 1991 Identification of a dominant negative form of the human estrogen receptor. Mol Endocrinol 5:1707-1715

39. Neff S, Sadowski C, Miksicek RJ 1994 Mutational analysis of cysteine residues within the hormone-binding domain of the human estrogen receptor identifies mutants that are defective in both DNA-binding and subcellular distribution. Mol Endocrinol 8:1215-1223

40. Miksicek RJ, Carlson KE, Hwang K-J, Katzenellenbogen JA 1995 Studies using fluorescent tetrahydrochrysene estrogens for in situ visualization of the estrogen receptor in living cells. Mol Endocrinol 9:592-604

41. Schweers LA, Frank DE, Weigel NL, Sanders MM 1990 The steroid-dependent regulatory element in the ovalbumin gene does not function al a typical steroid-response element. J Biol Chem 265:7590-7595

42. Fuqua SAW, Fitzgerald SD, Allred DC, Elledge RM, Nawaz Z, McDonnell DP, O'Malley BW, Green GL, McGuire WL 1992 Inhibition of estrogen receptor action by a naturally occurring variant in human breast tumors. Cancer Research 52:483-486

43. Kalkhoven E, Valentine JE, Heery DM, Parker MG 1998 Isoforms of steroid receptor co-activator 1 differ in their ability to potentiate transcription by the oestrogen receptor. EMBO J 17:232-243

44. Oñate SA, Tsai SY, Tsai M-J, O'Malley BW 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270:1354-1357

45. Xu J, Qiu Y, DeMayo FJ, Tsai SY, Tsai M-J, O'Malley BW 1998 Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science 279:1922-1925

46. Feng W, Ribeiro RCJ, Wagner RL, Nguyen H, Apriletti JW, Fletterick RJ, Baxter JD, Kushner PJ, West BL 1998 Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science 280:1747-1749

47. Vogel JJ, Heine MJS, Zechel C, Chambon P, Gronemeyer H 1996 TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. EMBO J 15:3667-3675

48. McInery EM, Tsai MJ, O'Malley BW and Katzenellenbogen BS 1996 Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator. Proc Natl Acad Sci (USA) 93:10069-10073

49. Andersson S, Davis DN, Dahlback H, Jornvall H, Russell DW 1989 Cloning, structure, and expression of the mitochondrial cytochrome P450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J Biol Chem 264:8222-8229

50. Jordan M, Schallhorn A and Wurm FM 1996 Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. Nucleic Acids Res 24:596-601

51. Klock G, Strähle U, Schütz G 1987 Oestrogen and glucocorticoid responsive elements are closely related but distinct. Nature 329:734-736

52. Gorman CM, Moffat LF, Howard BH 1982 Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2:1044-1051

53. Scatchard G 1949 The attractions of proteins for small molecules and ions. Ann NY Acad Sci 51:660-672

54. Laemmli UK 1970 Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680-685

55. Erickson PF, Minier LN, Lasher RS 1982 Quantitative electrophoretic transfer of polypeptides from SDS polyacrylamide gels to nitrocellulose sheets: a method for their re-use in immunoautoradiographic detection of antigens. J Immunol Methods 51:241-249

FIGURE LEGENDS

Fig.1. Comparison of ERα mRNA Splicing Variants and wt ERα Structure

The diagram depicts the various functional domains of the receptor and the exon sequences from which they are derived. The variants are referred to by deleted exon and are labeled in the left column. The size and molecular weight of each variant is predicted from the translational reading frame of the sequenced cDNA clones. The nuclear localization signal is circled. The regions encompassing the DNA- and hormone-binding domains are marked by darkened and striped boxes, respectively, where they are expressed. The AF-1 and AF-2 domains are indicated where they reside within the N- and C-termini, respectively, of the wild type receptor.

Fig. 2. Efficient *In Vivo* Expression of wt ERα and ERα Splicing Variants

Immunoblot analysis indicates that the molecular weight of each variant is consistent with its predicted translational reading frame. Samples containing 20 μg of protein from cell extracts were analyzed on a 10% SDS-polyacrylamide gel probed with the ERα-specific antibody, Mab-17. The figure is representative of three independent Cos7 transfection experiments.

Fig. 3. Gel Mobility Shift Assay to Assess the DNA-Binding Activity of ERα mRNA Splicing Variants

A gel mobility shift assay of lysate from 17β-estradiol treated, transiently transfected Cos7 cells confirms that wt ERα binds efficiently to a ^{32}P-labeled consensus estrogen response element. Confirmation that the indicated band represents an authentic ERα/DNA complex is provided by the ability of an ERα-specific monoclonal antiboldy (Mab-17) to supershift this complex (compare lane 4 with lane 3). In contrast, all of the splicing variants display strong defects in DNA-binding. In this representative assay, weak binding of ERΔE5 and ERΔE7 to the ERE can only be observed when their respective DNA complexes are stabalized by the addition of the ERα-specific antibody (lanes 12 and 16). Position of the antibody-supershifted complex is indicated by an asterix.

Fig. 4. Ligand Binding of ERα and ERα mRNA Splicing Variants.

A, Ligand-binding capacity was assessed by measuring the specific association of 10 nM ^{3}H-17β-estradiol with wt ERα and its splicing variants expressed in Cos7 cells, using the DCC method. Only wt ERα and the ERΔE3 variant demonstrate specific binding of estradiol. B, Confirming the ligand binding results, ERΔE3 and wt ERα are the only isoforms of the receptor observed to display specific cell staining using a fluorescent estrogen anologue (nitrile THC) to treat transiently transfected into Cos7 cells. C, Scatchard analysis shows that wt ERα and ERΔE3 have similar affinities for 17β estradiol.

Fig. 5. Localization of ERα and ERα Splicing Variants by Confocal Microscopy

Receptor isoforms were detected by immunofluorescence staining with an ERα-specific monoclonal antibody (Mab-17) and a rhodamine-conjugated secondary antibody. The upper left panel shows a representative field from control cells transfected with empty expression vector (pCMV4). Specific immunoreactivity can be observed in all other frames. Cells expressing wt ERα, ERΔE3 and ERΔE5 demonstrate strong nuclear staining in confocal sections. ERΔE2, ERΔE4, ERΔE6 and ERΔE7 are predominately localized to the cytoplasm. Bar, 10um.

Fig. 6. ERΔE3 and ERΔE5 Inhibit Transactivation of a consensus ERE Reporter by wt ERα in a Dose-Related Fashion

HeLa cells were cotransfected with 15 μg of pERE-TK-CAT reporter gene, 1 μg of wt ERα expression vector and increasing amounts of expression vectors for ERΔE3 or ERΔE5. The ratios of wt ERα to variant expression plasmid used in each transfection are indicated. The total amount of DNA in each transfection was held constant with the addition of empty expression vector, pCMV4. Reporter gene expression was normalized by measuring CAT activity in aliquots representing 100 μg of soluble protein.

Fig. 7. Transcriptional Activity of wt ERα and Receptor Variants

Only ERΔE3 and ERΔE5 supports CAT gene expression from the ovalbumin promoter, similar to wt ERα. HeLa cells were cotransfected with 10 μg of reporter (pOvalb-CAT) and 0.5 μg of the indicated ERα expression vector followed by 24 hours of hormone treatment in the presence of 5% charcoal-treated calf serum. Cultures were treated as indicated: vehicle control; $2x10^{-8}$M PMA; 10^{-8}M 17β-estradiol (E); E+PMA; and 10^{-7}M Tamoxifen (T) or T+PMA. CAT assays were normalized for protein. Values are expressed relative to vehicle-treated empty expression vector, pCMV4. Error bars represent the standard error of the mean of three independent experiments. As shown in the insert, none of the ERα splicing variants tested are strong activators of the ERE driven CAT reporter gene (pERE-TK-CAT) in HeLa cells.

Figure 1

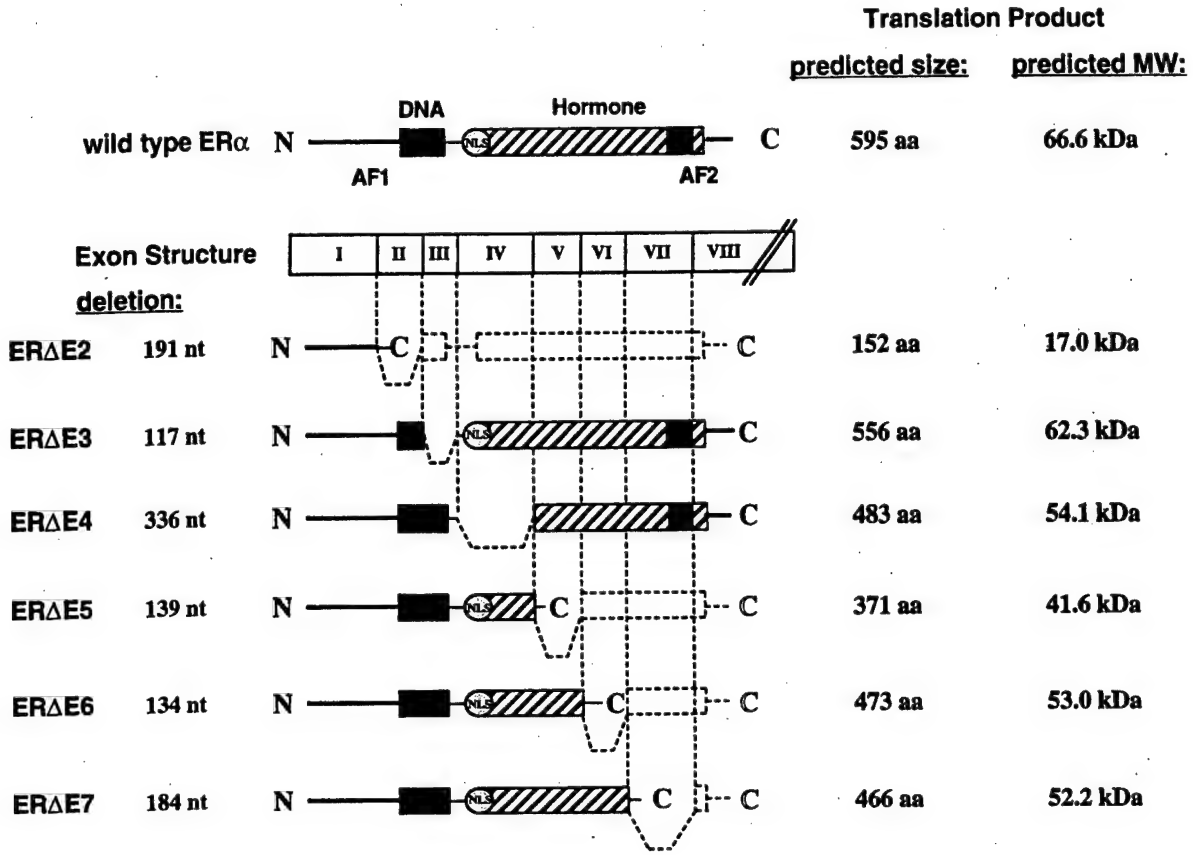

Translation Product
predicted size:
predicted MW:
wild type ERα
DNA
Hormone
N
C
NLS
AF1
AF2
595 aa
66.6 kDa
Exon Structure
I
II
III
IV
V
VI
VII
VIII
deletion:
ERΔE2
191 nt
152 aa
17.0 kDa
ERΔE3
117 nt
556 aa
62.3 kDa
ERΔE4
336 nt
483 aa
54.1 kDa
ERΔE5
139 nt
371 aa
41.6 kDa
ERΔE6
134 nt
473 aa
53.0 kDa
ERΔE7
184 nt
466 aa
52.2 kDa

Figure 2

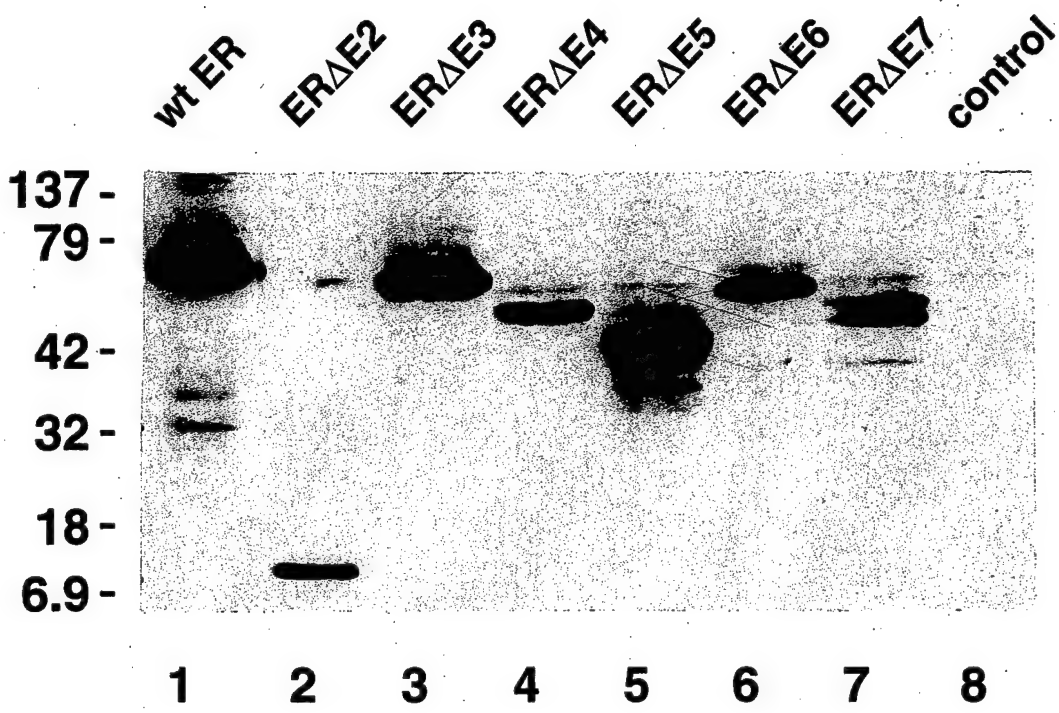

wt ER
ERΔE2
ERΔE3
ERΔE4
ERΔE5
ERΔE6
ERΔE7
control
137
79
42
32
18
6.9
1
2
3
4
5
6
7
8

Figure 3

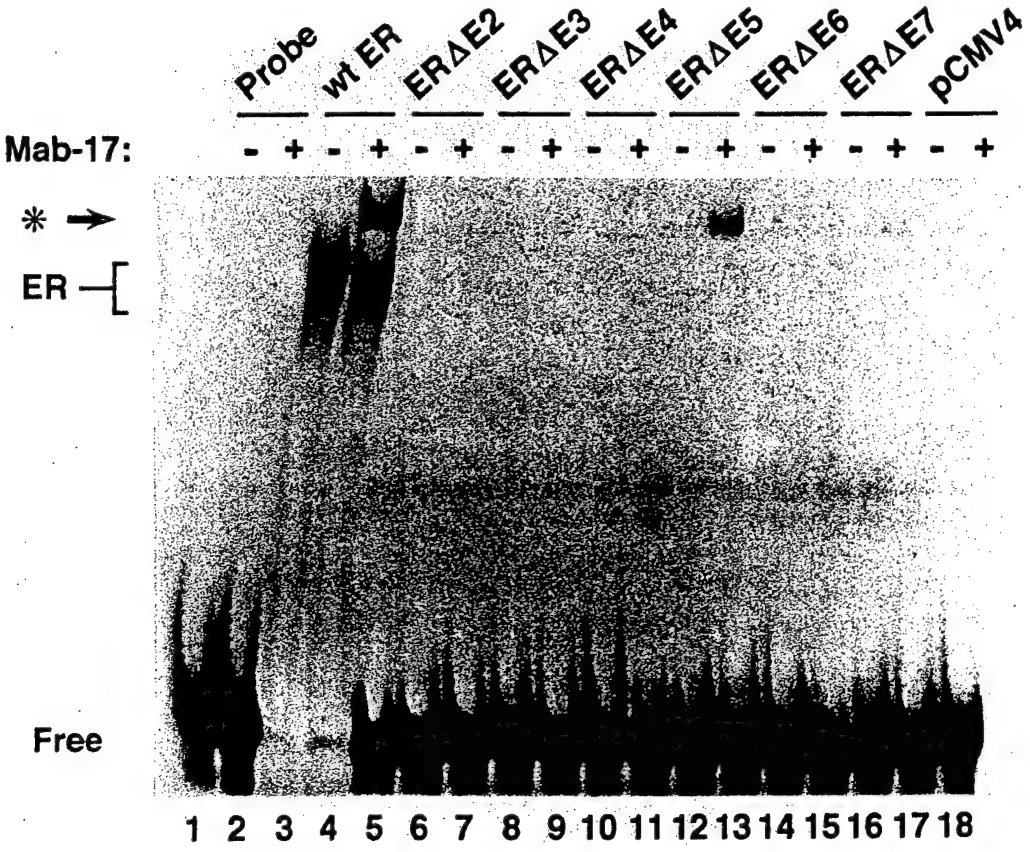
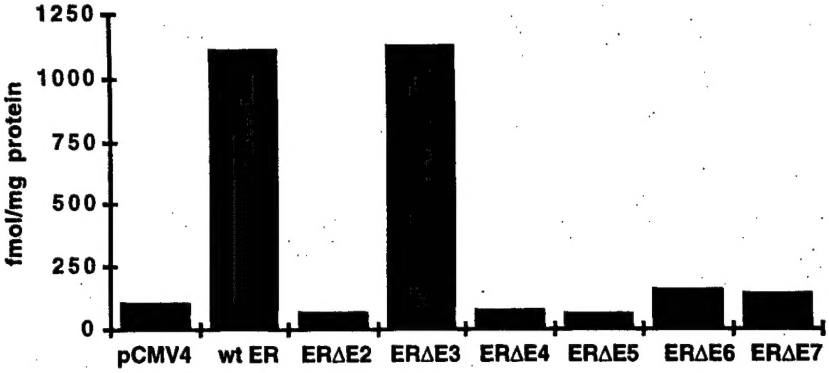
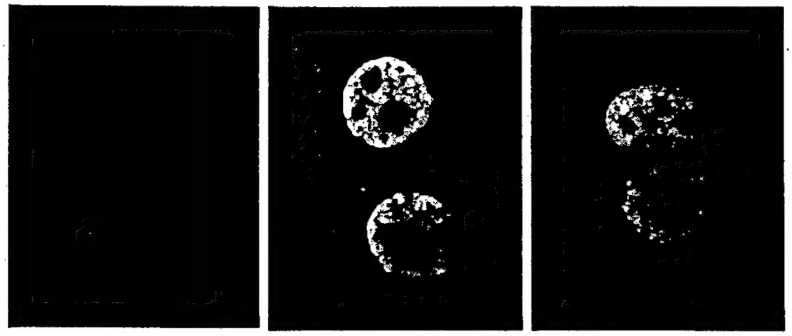

Probe
wt ER
ERΔE2
ERΔE3
ERΔE4
ERΔE5
ERΔE6
ERΔE7
pCMV4
Mab-17:
- + - + - + - + - + - + - + - + - +
*
ER
Free
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 4

A.


1250
1000
750
500
250
0
fmol/mg protein
pCMV4
wt ER
ERΔE2
ERΔE3
ERΔE4
ERΔE5
ERΔE6
ERΔE7

B.


pCMV4
wt ER α
ERΔE3

C.

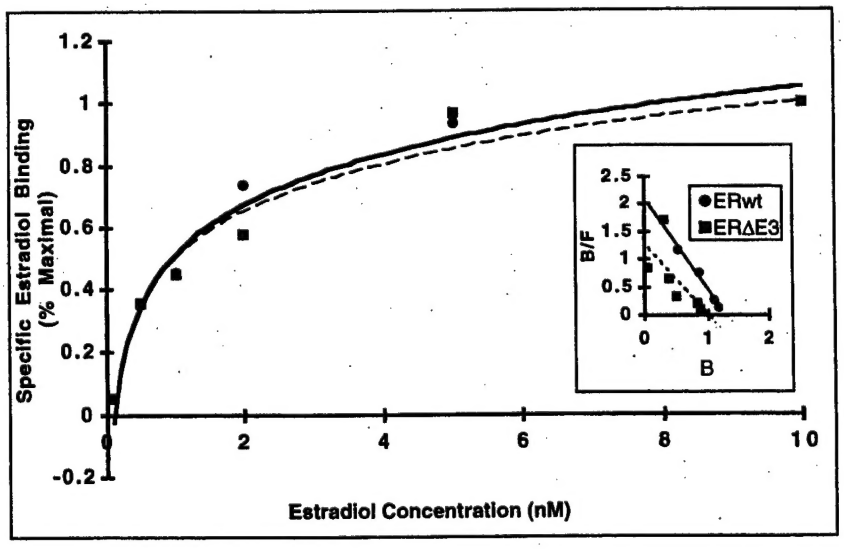

Specific Estradiol Binding
(% Maximal)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
2
4
6
8
10
Estradiol Concentration (nM)
B/F
2.5
2
1.5
1
0.5
0
0
1
2
B
ERwt
ERΔE3

Figure 5

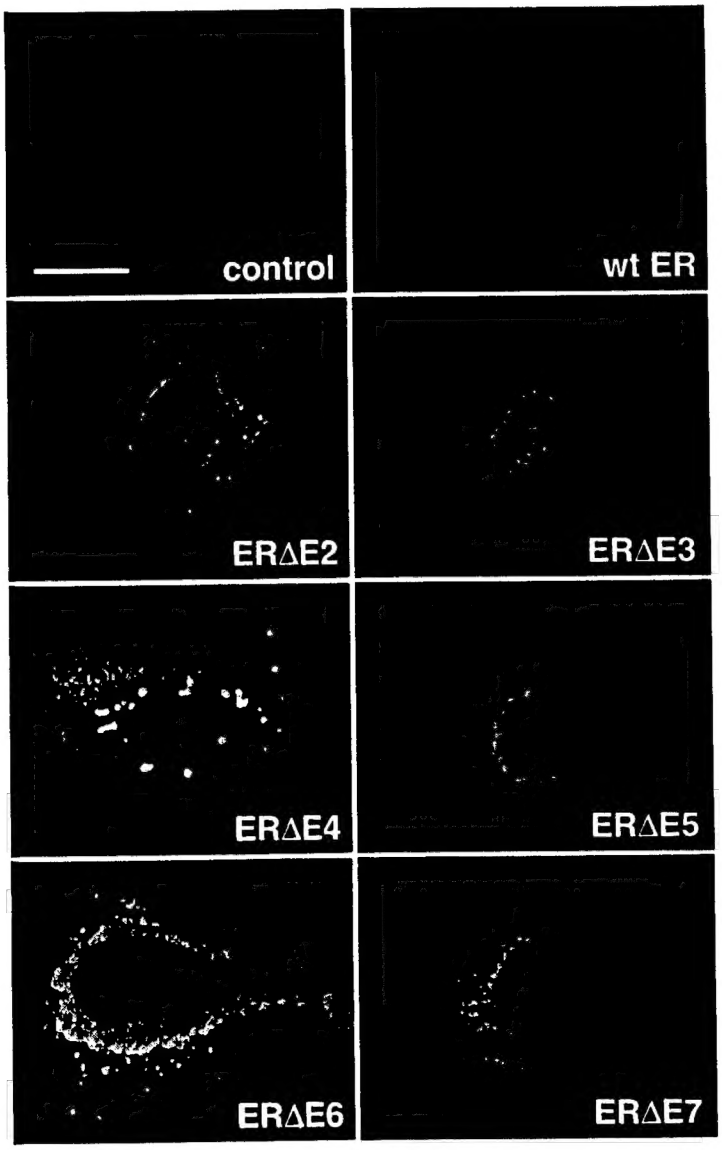


Figure 6

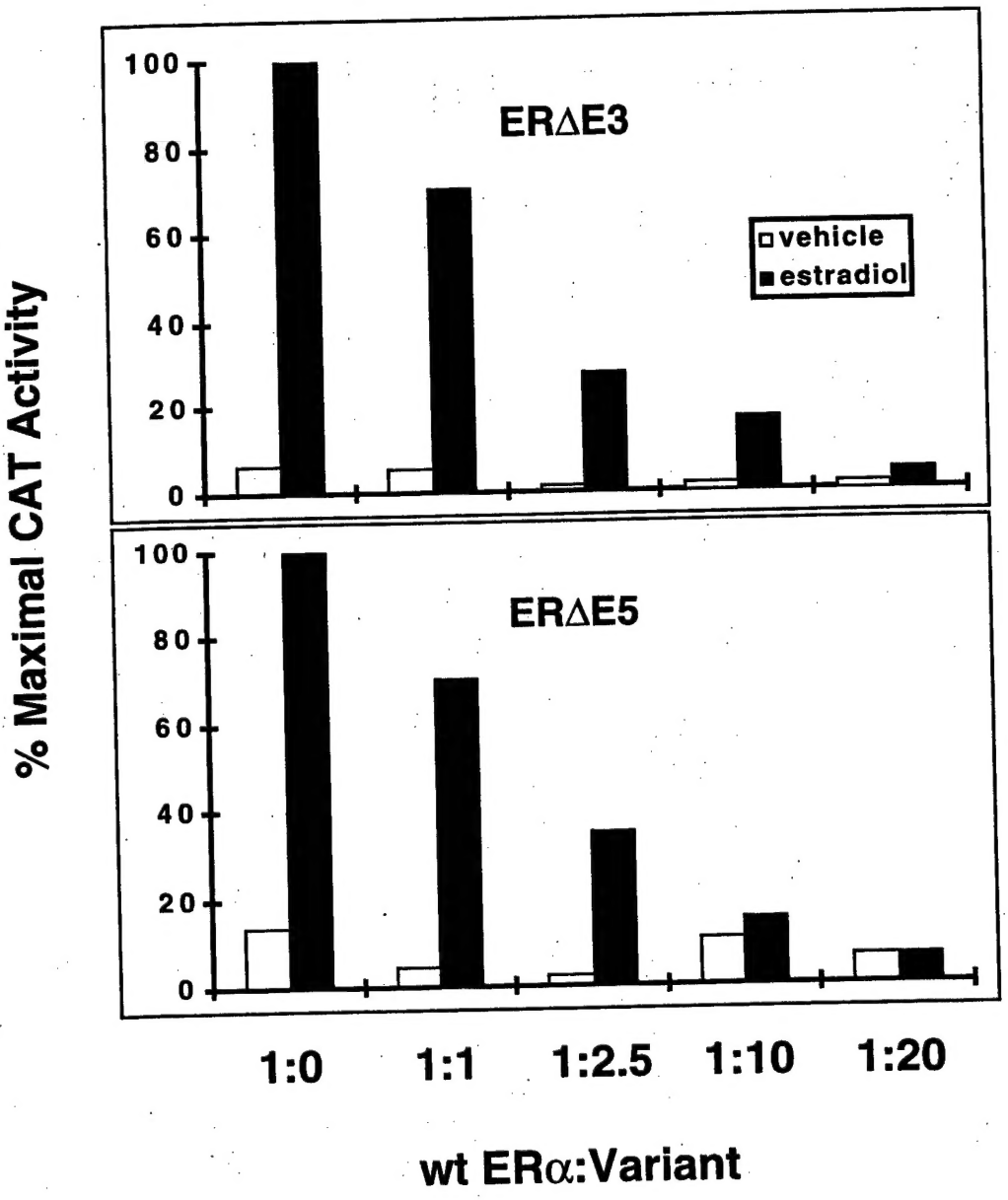
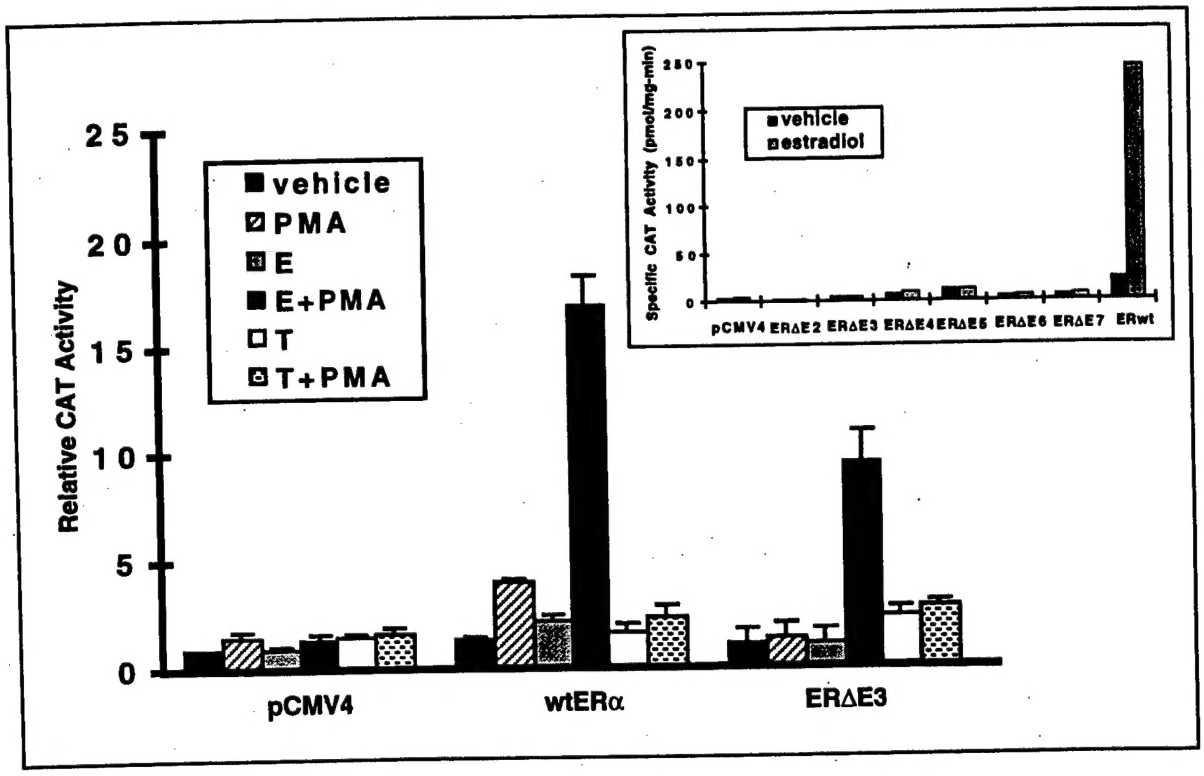

ERΔE3
ERΔE5
vehicle
estradiol
% Maximal CAT Activity
100
80
60
40
20
0
1:0
1:1
1:2.5
1:10
1:20
wt ERα:Variant

Figure 7


Relative CAT Activity
25
20
15
10
5
0
vehicle
PMA
E
E+PMA
T
T+PMA
pCMV4
wtERα
ERΔE3
Specific CAT Activity (pmol/mg-min)
250
200
150
100
50
0
vehicle
estradiol
pCMV4 ERΔE2 ERΔE3 ERΔE4 ERΔE5 ERΔE6 ERΔE7 ERwt